The Effect of Acute and Chronic Increases in Neuromuscular Activity on Gene Expression in

Small and Large Dorsal Root Ganglion Neurons: Healthy and Diabetic Rat

by

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ABSTRACT

Dorsal root ganglion (DRG) neurons are responsive to altered neuromuscular activity and play a role in diabetic peripheral neuropathy (DPN). Previous research has shown that chronic treadmill exercise training can delay the onset of DPN, and the expression of several genes are up-regulated in healthy whole DRG after acute voluntary wheel exercise. Understanding the effect of exercise on gene expression in small and large DRG neurons, in healthy and diabetic rats, may provide insight into mechanisms involved in changes to sensory processing associated with increases in neuromuscular activity and thermal hypoalgesia. We present evidence that small and large DRG neurons are differentially affected by exercise and diabetes.

We examined gene expression in samples of small and large neurons of the L4/L5 lumbar DRG, and their specific responses after exercise and diabetes, to identify potential molecular processes involved in activity-dependent changes. DRGs were collected 4 hours after the last exercise session, and RNA was isolated from samples of large and small DRG neurons following laser capture microdissection. Relative mRNA levels were determined using real-time polymerase chain reaction experiments.

In study 1, healthy adult rats received treadmill exercise for 1 or 17 weeks, or voluntary wheel exercise for 16 weeks. In study 2, STZ-induced diabetic rats received sedentary treatment or 15 weeks of voluntary wheel exercise. Behavioural testing of thermal latency response was performed on all animals in study 2. All control animals for both studies were healthy and sedentary. Gene expression analysis focused on ion channels, GABA and adrenergic receptors, glutamatergic receptors, serotonergic receptors, tyrosine kinase receptors, growth-related,

synaptic vesicle-related and pain-related genes because of their potential roles in nociception and proprioception.

In study 1, sedentary rats displayed higher expression in small relative to large neurons for 19 genes, higher expression in large compared to small neurons for 15 genes, and no difference in expression between small and large neurons for 12 genes, out of the 46 studied. In small and large DRG cell samples, acute treadmill exercise did not significantly alter the expression of mRNA for any genes examined. Following chronic treadmill or voluntary wheel exercise, gene expression responses in small and large DRG neurons varied considerably between types of exercise. In small DRG neuron samples, chronic treadmill exercise increased the expression of mRNA for 5HT1D and decreased expression for 5HT1F receptors. In large DRG neuron samples, chronic treadmill exercise decreased the expression of mRNA for 5HT1A and TrkC receptors, and Syn1. In small DRG neuron samples, chronic voluntary wheel exercise decreased the expression of mRNA for 5HT1D, TrkA and OPRD1 receptors and increased expression for GAP43. In large DRG neuron samples, chronic voluntary wheel exercise increased the expression of mRNA for 5HT3A and GAP43 and decreased expression for 5HT1D, Nav1.6, OPRD1, TrkA, TrkC and Syn1. Small and large DRG neurons respond differently to the duration and intensity of exercise. No clear pattern relating to the responsiveness of particular groups of expressed genes was apparent in any condition. DRG neurons seem to respond to chronic increases in neuromuscular activity. DRG neurons show a greater response to voluntary exercise, with less distance travelled and less intensity during the training period, compared to forced exercise.

In study 2, diabetes with and without voluntary wheel exercise did not significantly alter the expression of mRNA for any genes examined in large DRG neuron samples. Small DRG

neurons from diabetic sedentary animals contained higher 5HT1F and lower TrkB mRNA levels than those from healthy sedentary animals. In small DRG neuron samples, there was a decrease in CGRP mRNA in diabetic voluntary wheel exercised animals. Five weeks of diabetes resulted in prolonged withdrawal latencies in both sedentary and exercised rats. 8-11 weeks of diabetes resulted in a longer withdrawal latency in diabetic sedentary rats, but not exercised rats, compared to healthy sedentary rats. Voluntary wheel exercise lowered the level of 5HT1F transcript and prevented the decrease in TrkB receptors caused by diabetes. These genetic changes may be correlated with the prevention/reversal of thermal hypoalgesia associated with DPN.

Our results demonstrate that exercise affects expression of genes involved in activity-dependent neural plasticity differentially in small and large DRG neurons, and that these changes vary with prolonged and less intense periods of exercise, and with DPN. For the genes tested, small cells were more affected than large neurons in diabetic animals, whereas large neurons were more affected than small cells in healthy animals after chronic exercise. The results suggest potential new mechanisms for analgesia and the delay in diabetic peripheral neuropathy associated with exercise, among other nociceptive and proprioceptive implications.

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DEDICATION

This dissertation is lovingly dedicated to my parents, Maxwell and Ruby Paddock.

I appreciate your unconditional love and you always being there for me with encouragement and positivity. You are an inspiration to me on every level, and a true example of perseverance. Your faith in God, and your positive attitude towards life and struggles, have been a very powerful influence on me. You taught me to work hard and never give up, to do my best, to trust in God, and always smile, especially in the face of adversity. There is so much I could say, and yet no words can express, the deep gratitude and appreciation I feel to have been blessed with such amazing parents who freely give of themselves, with wise counsel, discipline, understanding, and the freedom and means to pursue my goals. I could not have completed this without you. For your love and unfailing support, I thank-you and I love you.

"Be strong and courageous and do the work. Don't be afraid or discouraged by the size of the task, for the Lord God, my God, is with you. He will not fail you or forsake you."

~ 1 Chronicles 28:20

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LIST OF ABBREVIATIONS

ADRA	adrenergic receptor
AHP	afterhyperpolarization
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ACTH	adrenocorticotropic hormone
BCAA	branch chain amino acid
BDNF	brain-derived neurotrophic factor
CAMKII	Ca2+/calmodulin-dependent protein kinase II
CFA	complete freunds adjuvant
cDNA	complementary deoxyribonucleic acid
CNR	cannabinoid receptor
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CREB	cAMP response element-binding protein
DOMS	delayed onset muscle soreness
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
EPSC	excitatory post-synaptic current
IGF	insulin-like growth factor
DPN	diabetic peripheral neuropathy
GABA	gamma-aminobutyric acid
GH	growth hormone

GluR	glutamate receptor
GDNF	glial cell line-derived neurotrophic factor
GAP	growth associated protein
Hsp	heat shock protein
iENF	intraepidermal nerve fiber
IP3	inositol 1,4,5-triphosphate
IB4	isolectin B4
Kv	voltage gated potassium channel
LTP	long-term potentiation
LDCV	large dense-core vesicle
LCM	laser capture microdissection
MAPK	mitogen-activated protein kinases
mRNA	messenger ribonucleic acid
MNCV	motor nerve conduction velocity
NCV	nerve conduction velocity
NGF	nerve growth factor
Nav	voltage gated sodium channel
NE	norepinephrine
NR1	NMDA receptor subunit
NMDA	N-methyl-D-aspartate receptor
NT	neurotrophin
OPRD	delta type opioid receptor
OPRM	opioid receptor mu subunit

PGC	peroxisome proliferator-activated receptor gamma coactivator
PCPA	para-chlorophenylalanine
PRL	prolactin
PKC	protein kinase C
PAG	periaqueductal gray
p75NTR	low-affinity neurotrophin receptor
(RT)/qPCR	quantitative real-time polymerase chain reaction
RMP	resting membrane potential
RQ	relative quantification
RNA	ribonucleic acid
RVM	rostral ventromedial medulla
SYN	synapsin
SNCV	sensory nerve conduction velocity
SUBP	substance P
STX	syntaxin
SNAP	synaptosomal-associated protein
SK/KCa	small conductance calcium-activated potassium channels
TRPV	transient receptor potential vanilloid
TGN	trigeminal ganglia neuron
TTX	tetrodotoxin
Trk	tyrosine related kinase receptor
STZ	streptozotocin
VGlut	vesicular glutamate transporter

5HT   serotonin

INTRODUCTION

Sensory neurons transmit information from the periphery to the central nervous system (CNS). Some sensory neuron cell bodies are located within dorsal root ganglia (DRG), and therefore they are also referred to as DRG neurons. mRNA synthesis occurs primarily in the nucleus, which is located in the cell body. Small and large DRG neurons are associated with nerve fibers that transmit different types of sensory stimuli. A change in gene expression at the DRG, can result in altered sensory transmission. This thesis comprises two major studies that examine small and large DRG neuron gene expression changes due to exercise in both a healthy and diabetic rat model.

DRG neurons are responsive to increased neuromuscular activity (Molteni et al, 2004), and are modified in diabetic peripheral neuropathy (DPN) (Low et al, 1989; Zochodne, 1996). Due to the previously demonstrated positive effects of exercise on diabetes in general, there is a case for suggesting beneficial effects on the diabetic neuron. We investigated the differences between voluntary and forced exercise, as well as acute and chronic exercise, in regards to small and large DRG neuron gene expression. Exercise causing gene expression changes could provide mechanistic insight into the phenomenon of exercise analgesia in healthy rats, in addition to thermal hypoalgesia associated with DPN.

The following five major topics are covered in this introduction: 1) DRG neurons – form and function 2) Exercise effects in the sensorimotor nervous system 3) DRG neurons are altered with diabetes 4) Purpose of and Rationale for the current study and 5) Genes of interest in the DRG.

1. DRG Neurons – Form and Function

DRG Neurons

Dorsal root ganglion (DRG) neurons have pseudounipolar morphology. The neuron cell body has a stem axon that bifurcates at the T-junction to form both central and peripheral axonal projections. The dorsal root branches at the central terminal in the spinal cord, whereas the peripheral nerve branches at the sensory ending innervating skin, muscle/joints, bone, and viscera. The peripheral nerve and dorsal root make up the conducting axon. Impulses are propagated from the periphery, past the DRG and into the spinal cord, conveying peripheral stimuli to the central nervous system (CNS) (i.e. 'through-conduction') (Amir & Devor, 2003). The following provisions ensure through-conduction of the signal: stem axon coiling around the soma to distance it electrically from the axon, and a decrease in axon diameter and short internodes on both sides of the T-junction making the nodes of Ranvier closer to each other (Cajal, 1911; Devor, 1999).

However, somata are electrically excitable, and although not required for sensory communication between the periphery and CNS, spikes travel from the periphery, up the stem axon and into the soma as well (Devor, 1999). One potential functional role for spike invasion of the soma is providing feedback from the periphery to the soma regarding cell metabolism required to address the functional requirements at the nerve ending (Devor, 1999). For instance, the feedback signal could be ongoing sensory impulse traffic, so that an increase in neuron firing would result in the soma acting to decrease excitability of the ending. In this case, the soma would modulate synthesis and transport of transduction and voltage channel proteins that determine afferent excitability. Moreover, since these molecules have various turnover rates, they have to be continually regulated. Thus, the provided electrical signal feedback would allow

the soma to alter sensory coding properties of the afferent, and ultimately, alter sensory signalling.

Furthermore, somata can also initiate electrical impulses. The thousands of cell bodies contained in one DRG have various complements of molecules they express which determine the cells' functional properties. For example, substances injected into the systemic circulation cause DRG neuron membrane receptors to generate an impulse discharge (Amir & Devor, 2003). The signal from DRG neurons can travel down the stem axon and propagate to the spinal cord and/or the periphery, affecting the release of neurotransmitters and other molecules at the terminals (Amir & Devor, 2003). Depending on the timing, an action potential initiated in the DRG could collide with a through-conduction action potential and extinguish it (Waxman, 2015). For example, a spike propagating distally can collide with and extinguish a signal propagating from the skin (Devor, 2015). However, most often, the signal from the DRG would likely add to the strength of the through-conduction signal (Devor, 2015). Other potential functional roles of an excitable soma membrane are repetitive firing by some cells on sustained depolarization, in addition to spontaneous firing in some neurons (Amir & Devor, 2003).

It is not clear what effect an action potential in the DRG has locally. DRG cell bodies are individually wrapped in a layer of satellite glial cells. Therefore, it is widely accepted that there are no known synapses present. Yet, almost all neuron somata are functionally linked via excitation during spike activity in neighboring neurons, although this effect is not extensive and only one neighbor could be affected for example. This phenomenon is called cross-excitation. Cross-excitation is evident in both intact (usually sub-threshold) and injured nerve impulse initiation (reach threshold and trigger spikes) (Devor, 1999). However, the functional role of cross-excitation is unclear. One theory is the metabolic regulation of receptor excitability in

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neurons that are not activated during normal daily activity, such as nociceptors (Devor, 1999). How cross excitation occurs is also not clear, but it seems that a neurotransmitter is released into the extra-cellular space causing potassium release from the cell. Another interesting fact about DRG cell bodies is that, unlike the rest of the peripheral nervous system (PNS), they are not protected by the blood-nerve barrier (BNB). Therefore, they have the potential to respond to blood-borne chemical signals via the chemoreceptors located on their somata (Amir & Devor, 1996; Devor, 1999). For instance, an increase in adrenaline leads to an increase in ongoing firing (Amir & Devor, 1996). This could be another source of cross-excitation. Furthermore, microvilli on each soma also increase the surface area available for exchange at the membrane (Devor, 1999).

Sensory nerve fiber classification. There are four main classifications of sensory peripheral nerve fibers: Aα (Ia and Ib), Aβ (II), Aδ (III) & C (IV) (Manzano et al, 2008). The alphabetical letter and Greek symbol classification was the first nerve fiber classification system proposed (For review please see Gasser, 1941). Later, the Roman numeral classification system was introduced (Lloyd, 1943). Type I fibers are 12-20um in diameter and innervate muscle. Type Ia and Ib fibers are the afferent axons for muscle spindles and Golgi tendon organs (GTO) respectively. Type II fibers are 6-12um and mainly found in skin, as well as spindle secondaries. Type III fibers are 3-4um, and Type IV fibers are unmyelinated. Both Type III and IV fibers innervate free nerve endings predominately in the skin (Parent, 1996).

In summary, most A-fibers are myelinated, have large nerve fibers and cell bodies, and fast conduction velocities (Harper & Lawson, 1985). However, the Aδ fibers are only thinly myelinated and have an intermediate conduction velocity (Gasser, 1941). C-fibers are non-myelinated, have small nerve fibers and cell bodies, and slow conduction velocities (Harper &

Lawson, 1985). The positive relationship between nerve diameter and conduction velocity was established by Gasser and Erlanger, 1927. Conduction velocity was related to cell type in DRG neurons by Harper and Lawson et al, 1985.

Each fiber type synapses within different areas of the spinal cord. Peptidergic C fibers project to lamina I neurons; Non-peptidergic C fibers project to inner lamina II neurons; Aδ fibers project to both lamina I and V neurons; Aβ fibers project to lamina III, IV, V and VI neurons; and Aα terminate on VI, VII and IX neurons (Grant & Robertson, 2004; Basbaum et al, 2009)

Figure 1: Spinal cord anatomy
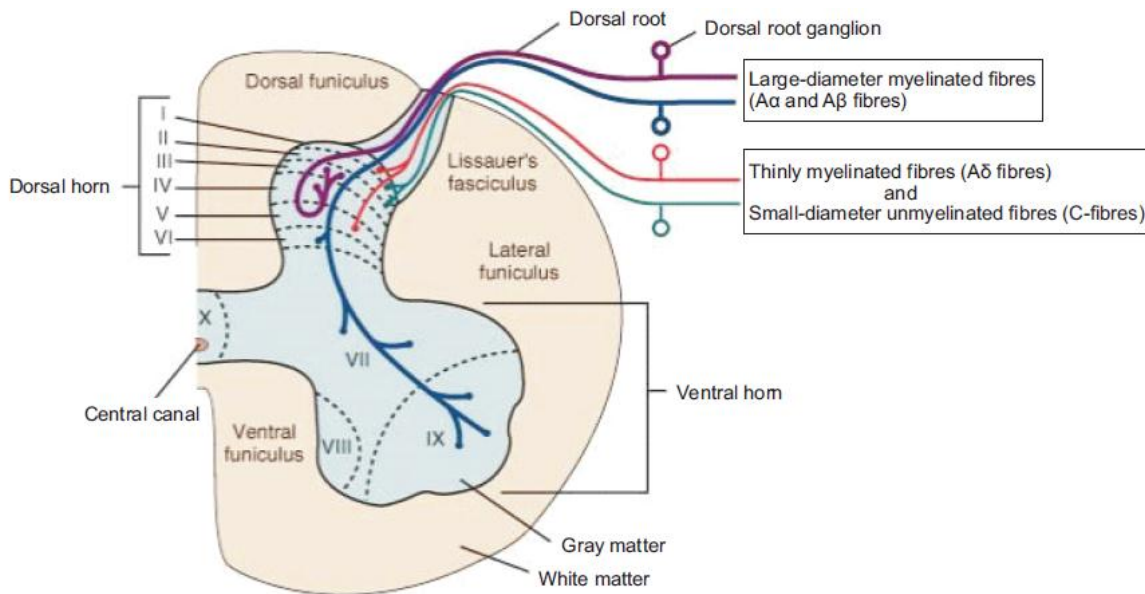
(Adapted from www.studyblue.com)

Peripheral receptor classification. Sensory receptors on peripheral terminals are involved in sensory transduction in response to various stimuli. The type of receptors present are dependent on location and type of stimuli transduced. For example, cutaneous receptors include mechanoreceptors, thermoreceptors, nociceptors, and chemoreceptors.

The mechanoreceptors found in the skin, which sense touch and respond to mechanical pressure or distortion, are: Pacinian corpuscles, Meissner's corpuscles, Merkel's discs, Ruffini endings and free nerve endings (please see Abraira & Ginty, 2013 for review). Cutaneous and subcutaneous mechanoreceptors are mainly innervated by Aβ fibers. Muscle and skeletal mechanoreceptors, also known as proprioceptors which sense limb position, are innervated mainly by Aα fibers. Muscle spindle proprioceptors are sensory nerve endings wrapped around muscle fibers that are activated by muscle stretch (Cooper & Daniel, 1956; Boyd, 1958). In joints, proprioceptors transmit joint position information (Gandevia et al, 2002). Thermoreceptors respond to temperature, nociceptors respond to damage or potential damage, chemoreceptors respond to chemical stimuli, and all three of these types of sensory receptors are free nerve endings.

Hence, each soma size associated with a particular fiber type generally relays different sensory information. Aα and Aβ fibers (i.e. associated with large DRG cell bodies) relay information from muscle and skeletal mechanoreceptors. Aβ and Aδ fibers (i.e. associated with medium DRG cell bodies) transmit information from cutaneous and subcutaneous mechanoreceptors. Aδ and C fibers (i.e. associated with small DRG cell bodies) transmit signals from nociceptors and thermoreceptors (Harper & Lawson, 1985).

Figure 2: DRG anatomy
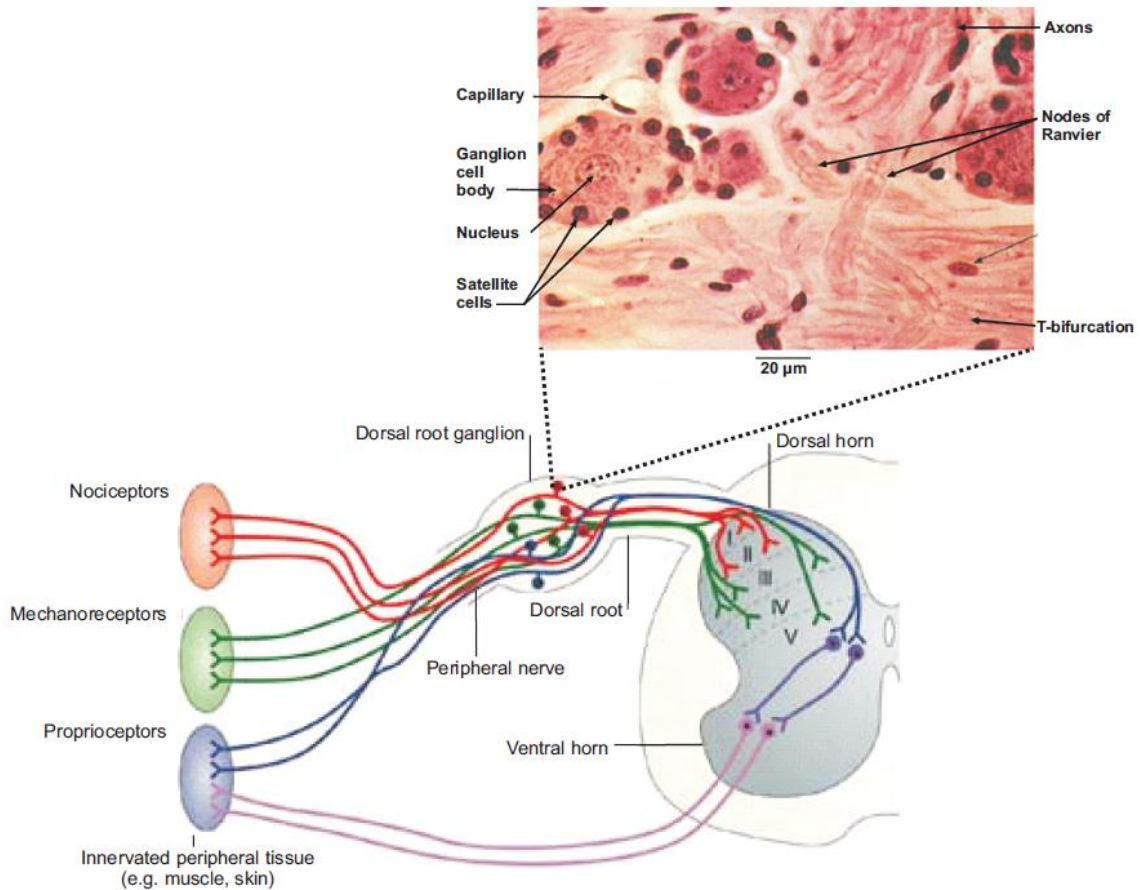
(Adapted from Caspary & Anderson, 2003)

Signal transmission. Sensory receptors transduce stimuli by generating action potentials that are propagated to the spinal cord via afferent fibers, which synapse with second order neurons that transmit the signal to the cerebellum or brain for processing, or to other spinal neurons. For instance, a knee tap to the patellar tendon inserted to the knee extensor muscle causes a stretch to the muscle activating stretch receptors that generate electrical impulses in the Aα sensory nerve fiber. The impulse is propagated to the spinal cord where it synapses with the α-motoneuron (quadriceps contraction), inhibitory interneuron (inhibits flexor motoneuron), and another interneuron that carries the signal to the cerebellum to coordinate the body movements

(Kandel et al, 2000). Another example is Pacinian corpuscle activation via pressure stimulation. The resultant deformation initiates a generator potential that, if it reaches threshold, creates action potentials at the first node of Ranvier, which is propagated toward the spinal cord. The higher the degree of deformation (ie. greater pressure), the higher frequency of impulses directed toward the primary somatosensory cortex for processing.

Messenger ribonucleic acid (mRNA) synthesis and the majority of energy metabolism, for both the soma and axon, occur in the soma of DRG neurons (Brown, 2003; Roy et al, 2005). For instance, transducer molecules located at the peripheral terminal are synthesized in the soma and transported down the axon. The same is true for other molecules, such as voltage-gated channels that encode generator potentials into a train of action potentials and signalling molecules at the spinal synapse.

The aforementioned information provides the rationale for examining mRNA presence and expression differences in DRG subpopulations, due to exercise and diabetes. In addition, other supporting evidence includes: 1) DRG neuron activation or de-activation of certain currents causes activation or inhibition at the spinal cord level (Yang et al, 2013) 2) certain proteins are formed in the sensory neurons and then transported to the primary afferent terminals (Towers et al, 2000) 3) receptors activated at the DRG cause downstream signalling of release of substances at both the central and peripheral terminals (Trevisani et al, 2007).

Why Separate Small And Large DRG Neurons?

The neuron size criteria we determined for laser capture micro-dissection (LCM) was 30um or less to classify small DRG neurons, and 40um or greater to classify large DRG neurons.

This classification is consistent with previous literature including work to establish DRG neuron cell body size in proportion to fiber type conduction velocity (Harper & Lawson, 1985). This relationship is generally true, although cell size can vary within fiber types. For instance, there is some overlap as a large cell body can be part of an Aδ fiber neuron, and a small cell body can be part of an Aβ fiber neuron (Harper & Lawson, 1985).

Studies have identified neuron size using several techniques in adult rat DRG. Small neurons ranged in size from 30um and less in diameter (Harper & Lawson, 1985; Scroggs & Fox, 1992; Fukuoka et al, 1998; Nicholson et al, 2003; Gong et al, 2014; Moraes et al, 2014; Nishida et al, 2014; Cheng et al, 2014). Large neurons were classified as greater than 40um in diameter (Harper & Lawson, 1985; Scroggs & Fox, 1992; Fukuoka et al, 1998; Nicholson et al, 2003; Keeler et al, 2012; Gong et al, 2014; Moraes et al, 2014; Nishida et al, 2014; Cheng et al, 2014). Considering there is also a medium, or intermediate, size of DRG neuron somata that range in size from 25-45um (Scroggs & Fox, 1992; Nicholson et al, 2003; Moraes et al, 2014; Nishida et al, 2014; Cheng et al, 2014), our criteria for small and large neurons were such as to exclude this population, as we were mainly interested in changes to nociceptive and proprioceptive transmission. Therefore, the size limitation of 30um or less for small, and 40um or greater for large DRG neuron cell bodies is consistent with the literature (Fukuoka et al, 1998; Gong et al, 2014; Moraes et al, 2014), and conducive to the purposes of this study.

We chose to isolate small and large neurons mainly because Molteni et al (2004) examined mRNA levels in healthy whole DRG of rats after exercise, but could not identify how each gene expression change would affect specific sensory transmission. The fiber type, and hence type of sensory information signaled, was not distinguished. However, by isolating small and large neurons, we could test to see gene expression changes in neurons associated with

particular fiber types (ie. nociceptors and proprioceptors). Furthermore, in the streptozotocin (STZ) rat model of diabetic peripheral neuropathy (DPN), mainly small cutaneous fibers die back first. Hence, using LCM of DRG neuron sub-types, we could investigate the small neuron response to exercise, as well as any changes in the large neurons. Therefore, isolating small and large DRG neurons is important in both studies of this thesis, and the results add novel information to the literature that is useful not only from a physiological perspective, but also pharmacologically and therapeutically.

Measurement of mRNA vs. Protein Content

Due to the small amount of RNA isolated using the LCM technique for single cell isolation, real-time quantitative (RT/q)-PCR was the most accurate and sensitive method to quantitate gene expression by directly measuring amplified mRNA levels in DRG neurons (Asplund et al, 2008). Because we wanted to investigate gene expression in small and large neurons in order to distinguish gene expression in these particular sensory pathways, and compare to whole DRG gene expression, LCM was required to isolate small and large cells. Hundreds of these cells were then pooled to get enough RNA to perform qPCR. mRNA levels for each gene were determined in small and large neurons, and gene expression differences in small and large neurons in response to exercise and diabetes were examined.

RNA is synthesized from DNA in the nucleus via transcription. mRNA is exported out of the nucleus for protein synthesis. Hence, mRNA is translated into protein in the cytoplasm. Thus, changes at the mRNA level can reflect changes in protein synthesis levels, if one assumes that a steady-state protein turnover condition is present. Therefore, if protein dictates function, then

changes in mRNA expression can indicate changes in function. Another reason to examine

mRNA instead of protein in DRG neurons, is that mRNA is synthesized in the cell body,

however, once the mRNA leaves the nucleus it can be transported as far as the distal axon

terminals before being translated into protein (Sigrist et al, 2000; Twiss et al, 2000; Amir &

Devor, 2003). Hence, the protein levels in DRG neurons alone may not represent the total

amount of functional protein translated. Furthermore, highly abundant protein will usually have

highly expressed mRNA (Vogel & Marcotte, 2012).

In a steady-state system, there is a high correlation between mRNA and protein in a wide

array of genes (Stahlberg et al, 2013; Greenbaum et al, 2003; Maier et al, 2009; Vogel et al,

2010; Schwanhausser et al, 2011; Gry et al, 2009; Asplund et al, 2008). The final level of a

protein in a cell depends on the rate of protein and RNA degradation, as well as the efficiency of

each step during synthesis. Gene expression is controlled by various degrees at each step:

transcription, RNA processing, RNA transport and localization, translation, and mRNA and

protein degradation (Alberts et al, 2008; Vogel & Marcotte, 2012). However, transcriptional

control is generally paramount in gene expression compared to post-transcriptional regulation in

terms of abundance (Alberts et al, 2008). It is common to use mRNA concentration as a proxy

for the concentration and activity of the corresponding protein, with the assumption that the

transcript abundances are the main determinant of protein abundances (de Sousa Abreu et al,

2009; Ramakrishnan et al, 2009).

In a perturbed system, correlations between mRNA and protein vary depending on the

perturbation and the gene, but generally an even higher correlation exists (Vogel & Marcotte,

2012; Lyons et al, 2012). With an increase in gene expression due to a perturbation, mRNA and

protein are well correlated (Lee et al, 2011). For example, after endurance exercise, mRNA and

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protein are highly correlated for the PGC-1α gene. In skeletal muscle, PGC-1α mRNA levels are significantly increased, followed shortly after by a significant increase in protein levels in both humans (Perry et al, 2010; Mathai et al, 2008) and rats (Wright et al, 2007; Terada et al, 2002; Woodrow et al, 2013). Increased PGC-1α protein results in increased muscle mitochondrial content (Spina et al, 1996), leading to improved oxidative capacity related to health and exercise performance benefits (Hood, 2001; Goodyear & Kahn, 1998). Therefore, the preceding increase in mRNA levels are indicative of the changes in function with endurance exercise.

Other studies also show an increase in neuronal gene expression with exercise (Perreau et al, 2005; Ferraiuolo et al, 2009; Molteni et al, 2004; Ying et al, 2003). Ying et al. (2003) showed an increase in NT3 mRNA and protein in muscle and spinal cord dorsal horn after voluntary wheel exercise leading to structural and functional changes in both the PNS and CNS. Molteni et al. (2004) showed increases in mRNA in the DRG related to increased neurite extension after exercise. Ferraiuolo et al (2009) showed that exercise increases BDNF transcripts in neurons and muscles resulting in enhanced responsiveness of neurons and activity of pathways signalling cell transport and remodelling. Other transcripts were also increased involving structural and functional alterations to the neuromuscular system such as synaptic reorganization and electrophysiological changes leading to optimized neural transmission. Other examples of highly correlated changes in mRNA and function in a perturbed system are discussed in the following section on pain.

In summary, mRNA levels are important to measure and the most suitable and possible measurement for our study. From these results, we can suggest functional implications. For instance, an increase in neuromuscular activity causing transcriptional change of neuronal membrane proteins, could result in altered biophysical properties of neurons (Gardiner et al,

2006), which can influence neuromuscular performance. In Study 2, we can base these suggestions on the behavioral data collected as a functional measure of change.

Pain Overview

Nociceptive pain. Normal pain, also known as nociceptive pain, involves the detection of damaging or potentially damaging noxious stimuli by nociceptors and the subsequent unpleasant sensation. This type of pain is necessary as a protective mechanism to avoid injury, as well as to allow healing after an injury has occurred. Hence, nociceptive pain is adaptive in nature and is evoked by high-intensity stimuli (Costigan et al, 2009). There are three groups of nociceptors: 1) peptidergic unmyelinated C-fibers 2) non-peptidergic unmyelinated C-fibers and 3) thinly myelinated Aδ-fibers, that can innervate skin, muscle, bone/joints, and organs. Aδ fibers convey acute, well-localized fast or first pain, whereas C fibers mediate slow or second pain (Dubin & Patapoutian, 2010).

Nociception is the "neural process of encoding and processing noxious stimuli" (Dubin & Patapoutian, 2010). Noxious stimuli activate high-threshold nociceptors that send a pain signal to laminae I and II of the spinal cord dorsal horn (Latremoliere & Woolf, 2009), where projection neurons send the signal to the brainstem and brain to be processed, as well as to other spinal cord neurons that are involved in the withdrawal reflex (Woolf, 2010). Nociceptors release signalling molecules from their central terminals that generate excitatory postsynaptic currents (EPSCs) and excite 2nd order neurons to transmit the pain message to higher order neurons. Fast and slow synaptic transmission is accomplished by glutamate and neuropeptides respectively (Dubin & Patapoutian, 2010).

Hence, normal pain perception requires information from the periphery to be sent to the brain, and transmission can be influenced at any level (ie. nociceptor, projection neurons, interneurons, and ascending and descending pathways).

There are different types of pain stimuli including mechanical, thermal and chemical. Noxious stimuli is detected and transduced into electrical impulses by pain sensor molecules on the peripheral terminal membrane (Foulkes et al, 2008). More specifically, noxious stimuli are transduced via ion flux, creating the receptor potential through direct or indirect (2nd messenger) modulation of ion channel activity (Dubin & Patapoutian, 2010). This change in membrane potential at the peripheral terminal activates voltage-gated ion channels, generating an action potential(s) that encodes the intensity of the stimulus applied within the receptive field of the nociceptor (Dubin & Patapoutian, 2010). Excitability and firing behaviour of nociceptors depend on the particular complement of voltage-gated channels present. For example, voltage-gated sodium channel (Na_v) 1.8 is essential for mechanical, cold and inflammatory pain, but not neuropathic pain or heat sensing (Abrahamsen et al, 2008). Following the transduction of noxious stimuli, the action potential is conducted from the peripheral to the central terminal of the nociceptor by sodium (Na_v1.8 and 1.9) and potassium (TREK-1, TRAAK, and K_vs) channels located along the axon (Foulkes & Wood, 2008; Amir & Devor, 2003).

Most nociceptors are polymodal (i.e. can sense and respond to mechanical, thermal and chemical stimuli), however, some have specific receptors that only transduce one type of stimulus (Foulkes et al, 2008). The main identified channels, transient receptor potential (TRP) channels, that participate in noxious stimuli transduction are TRPV1 for heat, TRPM8 for cold, TRPA1 for chemical, and the transduction channel for noxious mechanical stimuli is unknown (Basbaum et al,2009; Dubin & Patapoutian, 2010; Foulkes et al, 2008). The molecular basis for

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mechanical transduction is not clear. However, it is thought that pressure opens a mechanosensitive cation channel to elicit depolarization (Dubin & Patapoutian, 2010).

Synaptic transmission in the spinal cord is carried out mainly by glutamate and neuropeptides released from the afferent terminal, and signalling through their receptors on second order neurons (Foulkes et al, 2008). Electrical activity and molecular signalling in the DRG activate second messenger cascades that result in transcription of molecules that are then transported to both the central and peripheral terminals where they are released out of the terminal or inserted into the membrane to regulate synaptic strength and excitability (Woolf & Costigan, 1999).

The spinothalamic tract carries projection neuron signals to the thalamus and onto the somatosensory cortex (Basbaum et al, 2009). Collaterals go to the PAG, cerebellum, insula (Schweinhardt & Bushnell, 2010) and amygdala (Ossipov et al, 2010). Projection neurons also go to the parabrachial nucleus via the spinoreticular tract, onto the hypothalamus or amygdala, ending in the insular, cingulate (Basbaum et al, 2009) or prefrontal cortices (Schweinhardt & Bushnell, 2010). Ascending collaterals also go to the rostral ventromedial medulla (RVM) (Basbaum et al, 2009) and dorsal reticular nucleus (DRt) (Ossipov et al, 2010). Therefore the ascending pathways and associated brain regions are involved in pain processing resulting in pain perception. For example, location and intensity of painful stimuli is processed by the somatosensory cortex, whereas the affective component of pain is processed by the limbic areas (Basbaum et al, 2009). The supplemental motor area and basal ganglia are also involved in pain processing (Schweinhardt & Bushnell, 2010).

Contrastingly, descending pathways are involved in pain modulation (Schwerinhardt & Bushnell, 2010). For instance, descending feedback from cortical sites, the amygdala,

hypothalamus, periaqueductal gray (PAG), and locus coeruleus (LC) have their final synapse at the RVM before projecting to the spinal cord dorsal horn where the input regulates (ie. inhibit or facilitate) transmission of nociceptive information (Heinricher et al, 2009; Schweinhardt & Buhsnell, 2010; Ossipov et al, 2010; Foulkes et al, 2008). The RVM contains "on"and "off" cells. The pain threshold varies with the balance between these cells. For example, more "on" cells firing produces a pronociceptive effect, while more "off" cells firing results in antinociception (Schweinhardt & Bushnell, 2010). Additionally, some DRt neurons project to the dorsal horn and are thought to be facilitatory (Heinricher et al, 2009). The interacting brain regions that form loops and systems and are involved in pain processing and modulation make up the "pain matrix" (Ossipov et al, 2010).

Afferent nociceptive synapses can also be influenced by non-nociceptive neurons. Large fibers, that terminate in deeper laminae but send collaterals to inhibitory interneurons in LII, synapse onto the projection neurons (Latremoliere & Woolf, 2009).

Altered sensations associated with various pain states are hyperalgesia and allodynia. Hyperalgesia is an increase in pain perception (ie. exaggerated response) due to noxious stimulation. Allodynia is pain due to a stimulus that is not normally painful (ie. pain due to innocuous stimulation). Spontaneous pain occurs with no external stimulus at the peripheral terminal.

Inflammatory Pain. A second classification of pain is inflammatory. Inflammatory mediators, which are signalling molecules (i.e. serotonin, histamine, NGF), are released from non-neuronal cells (ie. mast cells, macrophages, platelets, immune cells, keratinocytes) and from the activated nociceptor itself (i.e. CGRP, SubP) at the peripheral nociceptor terminal in response to tissue damage or other causes of inflammation (McMahon & Koltzenburg, 1990; Cheng et al,

2008). Although this type of pain can be activated by both low (ie. Aβ fiber inputs) and high-threshold stimuli, it is still adaptive as its role is to protect by discouraging contact or movement, thereby promoting repair (Woolf, 2010; Costigan et al, 2009). In contrast to nociceptive pain, inflammatory pain can be activated without any stimuli (ie. spontaneous pain) and normally non-painful stimuli (i.e. Aβ induced tactile allodynia), and there can also be an exaggerated response to noxious stimuli (ie. C and Aδ induced hyperalgesia) (Costigan et al, 2009).

Signalling molecules, such as neuropeptides, when released from the peripheral terminal causes vasodilation and plasma extravasation (Gamse & Saria, 1985; Brain & Williams, 1989; Brain et al, 1992). However, inflammation-evoked release of mediators at the periphery causes an immediate post-translational change in transduction molecules and other ion channels that activate PKC and PKA signalling, leading to phosphorylation of Nav's at the nociceptor peripheral terminal for example (McMahon & Koltzenburg, 2006; Basbaum et al, 2009). The aforementioned response results in spontaneous pain and/or pain hypersensitivity, with an increase in responsiveness and a decrease in threshold (i.e. peripheral sensitization) (Woolf & Decosterd, 1999). The response of transcriptional changes (ie. minutes to hours) at the DRG is caused by an increase in activity at the DRG as well as retrograde transport of signal molecules from the periphery to the DRG, such as TrkA/NGF (Mantyh et al, 2011). The increased synthesis of neuropeptides, neurotrophins, receptors and ion channels leads to the anterograde transport of these molecules from the DRG to the spinal cord and peripheral terminal membranes (Mantyh et al, 2011; Woolf & Costigan, 1999).

At the peripheral end, the result is modification in both the propagation of impulses to the dorsal horn (i.e. increased Na and Ca and decreased K currents and/or number of channels) and the fibers' response to sensory stimuli (.ie. increase in TRPV1), which can alter the phenotype

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(i.e. large DRG neurons can now express SubP and BDNF) (Latremoliere & Woolf, 2009) as well as potentiate the system (i.e. increase in TRPV1 leads to thermal hyperalgesia) (Dubin & Patapoutian, 2010; Mantyh et al, 2011; Woolf & Decosterd, 1999). Hence, inflammation can alter the sensitivity of action potential transmission and transduction by nociceptors resulting in an increase in neuronal sensitivity and hyperalgesia (Foulkes et al, 2008).

At the central end, the result of an increased concentration of signalling molecules (i.e. Glutamate, BDNF, SubP, CGRP) binding to post-synaptic receptors, is change in transcriptional activity, removal of the Mg block of NMDA receptors, facilitating synaptic transmission and strong depolarization of the projection neuron (Mantyh et al, 2011). Additionally, projection neurons now express the glutamate receptor GluR1, as opposed to GluR2, in the basal state, leading to an increase in Calcium influx on activation (Latremoliere & Woolf, 2009). Furthermore, microglia are activated which release factors that decrease inhibitory and increase facilitatory currents (Latremoliere & Woolf, 2009). Each of these changes contribute to central sensitization caused by inflammation.

Neuropathic pain. A third classification of pain is neuropathic. Neuropathic pain is pathological, as it is a disease state caused by damage to the peripheral (i.e. neuropathy) and/or central (i.e. spinal cord injury (SCI)) nervous system (Woolf, 2010). Neuropathic pain is evoked by both low and high intensity stimuli, and ectopic action potential generation, which can potentially elicit central and peripheral amplification (Costigan et al, 2009), leading to spontaneous pain and pain hypersensitivity. For example, Na_v's may accumulate in injured nerve fibers causing an increase in spontaneous firing or alteration in conduction or neurotransmitter properties, leading to pain. This type of pain is maladaptive, and is commonly persistent, even in the absence of a lesion or disease (Costigan et al, 2009).

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The type and location of damage determines the somatosensory system response. For instance, in the event of peripheral nerve injury, there may be injured and intact sensory neurons with varied responses. Nerve injury can cause the loss of synaptic connections and the formation of new ones, for instance, a loss in C-fiber central terminals and the sprouting of Aβ-fibers to LI and LII to make contact with nociceptors. Mediators are released from microglia, Schwann cells, immune cells and both damaged and non-injured axons (Cheng et al, 2008; Latremoliere & Woolf, 2009; Costigan et al, 2009). Disinhibition occurs via decreased descending inhibitory control, inhibitory interneuron apoptosis, and a switch in chloride gradients. Disinhibition, along with the following changes, result in increased excitability: increased transcription and trafficking of ion channels that alter membrane potential, changes in ion channel threshold and kinetics, increased descending facilitation from the RVM, large fibers begin to express SubP and BDNF, and ectopic impulse generation (i.e. in intact and injured nociceptive and low-threshold neurons and neuromas) and transduction (i.e. increased TRPV1 threshold resulting in increased sensitivity to external stimuli in injured neurons) (Costigan et al, 2009; Latremoliere & Woolf, 2009; Woolf, 2010).

In summary, a nerve lesion can cause a change in structure and function of the somatosensory nervous system, leading to spontaneous pain and the amplification of both noxious and innocuous stimuli (Costigan et al, 2009).

Dysfunctional pain. A fourth classification of pain is dysfunctional. Dysfunctional pain is also pathological, being a disease state of the nervous sytem, yet there is no lesion, inflammation, or identifiable noxious stimuli (Costigan et al, 2009). Despite normal tissue and nerves, there is spontaneous pain and pain hypersensitivity which is maladaptive (Woolf, 2010). Hence, pain can be present with no, normally non-painful and painful stimuli (ie. both low and

high threshold). Dysfunctional pain usually involves a disturbance in the balance of excitation and inhibition centrally, both peripheral and central amplification, altered sensory processing, and a decrease in pain threshold (Costigan et al, 2009).

Sensitization. Briefly mentioned above, sensitization is the amplification of the response to stimuli when the sensitivity of the nervous system is disturbed (Costigan et al, 2009), causing an increase in pain perception (Dubin & Patapoutian, 2010). Nociception, nerve injury and inflammation can all lead to sensitization. For example, repeated or intense noxious stimulation results in a decrease in threshold for activation of the nociceptor and amplification of the response to previously subthreshold inputs, making the system "hyperalert" and aiding in nociceptive pains' protective role (Latremoliere & Woolf, 2009).

Central sensitization. The particular sensation experienced due to central sensitization (CS) is based on the type of external stimulus provided. More specifically, a sensitized central pathway can be caused by innocuous (i.e. activates low threshold neurons) or noxious (i.e. activates nociceptive neurons) peripheral stimuli, or lack thereof, which leads to secondary allodynia, secondary hyperalgesia and spontaneous pain, respectively (Costigan et al, 2009). Three main factors leading to CS are: 1) increased Glutamate (i.e. NMDA) receptor signalling 2) disinhibition 3) microglial activation.

When pre-synaptic afferent terminals release signaling molecules during CS, specifically glutamate, there is an increase in NMDA receptor signalling (ie. no Mg^{2+} block), resulting in increased intracellular calcium and thus activation of calcium-dependent signalling pathways (ie. PKC and CaMKII) and second messengers (ie. Erk1/2) (Basbaum et al, 2009; Latremoliere & Woolf, 2009). This post-synaptic signalling displays activity-dependent post-translational immediate modifications. Once activated, PKA and Erk1/2 lead to transcription of several

molecules (ie. TrkB, NK1), as well as promoting trafficking of glutamate receptors to the

membrane, which further strengthens the synapse (Latremoliere & Woolf, 2009). Once

phosphorylation occurs, there is a decrease in threshold and change in activation kinetics of

NMDA/AMPA receptors leading to increased membrane excitability and enhanced synaptic

efficacy (Woolf & Costigan, 1999). Likewise, peripheral inflammation causes a switch in active

glutamate receptors allowing calcium entry, and also causes other membrane receptors (ie.

mGluR, NK1, B2, CGRP1) to release intracellular calcium stores, both leading to enhanced PKC

and CAMKII signalling and a stronger excitatory synapse (Latremoliere & Woolf, 2009).

Therefore, more intracellular calcium corresponds to a stronger excitatory synapse (Latremoliere

& Woolf, 2009). The consequent increase in excitability of the projection neuron corresponds to

facilitation in the transmission of pain information to the brain (i.e. hyperalgesia) (Basbaum et al,

2009).

Activation of inhibitory interneurons that normally release GABA/Glycine (GLY) onto

projection neurons, and Aβ synaptic glutamatergic interneurons that synapse onto projection

neurons, decrease excitability and modify pain transmission (Basbaum et al, 2009). However,

this inhibition can be lost due to injury via decreased release or activity of GABA/GLY, leading

to increased depolarization and excitation of projection neurons, eliciting the sensations of

hyperalgesia and allodynia (ie. Aβ induced pain transmission) (Guo & Hu, 2014).

Activated microglia release cytokines and BDNF that bind to projection neurons,

increasing excitability and pain transmission (Basbaum et al, 2009).

Therefore, multiple cellular processes lead to the altered functional properties of

nociceptive neurons and circuits associated with CS. These changes produce increased

membrane excitability, facilitation of synaptic strength of sensory and dorsal horn neurons, as

well as decreased inhibitory transmission. Therefore, pain is no longer coupled to noxious peripheral stimuli as CS produces pain hypersensitivity by changing the sensory response elicited by innocuous inputs. Basically, anything leading to increased membrane excitability of the post-synaptic neuron can amplify the response to stimuli and increase processing of pain messages (i.e. elicit CS).

Peripheral sensitization. During peripheral sensitization (PS), both non-noxious and noxious stimuli can activate sensitized peripheral nociceptive terminals causing primary allodynia and hyperalgesia, respectively (Costigan et al, 2009). Noxious stimuli are not required to generate pain since cell bodies change in expression and trafficking of proteins, hyperexcitable axons generate spontaneous action potentials, and the nociceptor peripheral terminals change to become sensitized during inflammation. Peripheral tissue injury and/or nerve damage can produce and release inflammatory mediators that can, along with spontaneous electrical activity, stimulate receptors on terminals, axons and soma. The subsequent activation of protein kinases can lead to rapid post-translational regulation via phosphorylation of TRPV1 and Nav's causing hyperactivity. Kinase activation also leads to transcriptional and translational regulation, resulting in increased synthesis of pro-nociceptive proteins (ie. TRPV1, Nav, Cav, BDNF, SubP, CGRP, etc). Persistent protein synthesis in sensory neurons causes sustained nociceptor hypersensitivity, and usually requires ongoing pathology.

In summary, increased synthesis and activity of ion channels and neuromodulators increases sensitivity (i.e. ↓ threshold and ↑ responsiveness) and excitability of the nociceptor, thereby inducing PS (Schmidt et al, 2009).

2. Exercise Effects in the Sensorimotor Nervous System

The sensorimotor nervous system can be modulated by several factors including aging (Luff, 1998), injury (Seburn & Cope, 1998), diseases such as amyotrophic lateral sclerosis (ALS) (Foran & Trotti, 2009) and multiple sclerosis (MS) (Fischer et al, 2013), and changes in activity (Dishman RK et al, 2006). Changes caused by an increase in regular daily physical activity, for example endurance training, have not been extensively researched but are apparent in motoneurons (Gardiner et al, 2006), spinal cord (Krityakiarana et al, 2010), brain (Neeper et al, 1996), and DRGs (Molteni et al, 2004). Exercise has many beneficial effects demonstrated by enhanced cognition with aging (Kohman et al, 2011; Aguiar et al, 2011), improved rehabilitation injury recovery (Rossignol & Frigon, 2011), and treatment and prevention of many disease states (Lau et al, 2011).

PNS adaptations to exercise are less studied compared to the CNS changes. Change to the responsiveness of DRG neurons with exercise may be revealed in gene expression changes in proprioceptors, nociceptors and other neurons as well. Functional implications of changes in gene expression at the DRG could be exercise analgesia, as well as preventing or delaying sensory nerve function loss associated with DPN. An understanding of how the function of sensory neurons is modulated by exercise in both normal and diseased populations is important to determine mechanisms involved in how the somatosensory nervous system, and more specifically the PNS, responds to exercise. It is also important in order to create injury rehabilitation protocols, and treatment and prevention strategies for abnormal nervous system conditions.

Exercise Effects in the Brain

Exercise affects overall health and brain function. The majority of the effects of exercise on the nervous system have been studied in the brain, and more specifically the hippocampus. This area is central to learning and memory, is plastic throughout the lifespan, and is modified by physical activity (Van Praag et al, 1999). Several mechanisms of action have been proposed. For example, running wheel exercise leads to synaptic plasticity as evidenced by improved Morris water maze performance and learning shown by increased long term potentiation (LTP) within the dentate gyrus of young mice (Van Praag et al, 1999). Furthermore, as wheel running speed increased, there was a corresponding increase in hippocampal cell firing (Czurko et al, 1999). Voluntary exercise prevented negative morphological and behavioral deficits in aged mice (Van Praag et al, 2005), and cognitive deficits associated with this brain area were attenuated by exercise (Fordyce & Wehner, 1993; Lautenschlager & Almedia, 2006).

Exercise activates cellular and molecular cascades that support and maintain changes in the brain such as neurogenesis (Van Praag et al, 1999), neuroadaption (Johansson et al, 1997) and neuroprotection (Kramer et al, 2006). Imaging studies showed increased cerebral blood volume correlated with increased cardiopulmonary and cognitive function due to exercise-induced neurogenesis (Pereira et al, 2007). Most genes found to be regulated in the brain by exercise were related to synaptic structure and plasticity (Cotman & Berchtold, 2002).

Commonly explored molecular mechanisms of action of exercise-induced brain changes involve BDNF and its up and downstream modulators. BDNF and NGF are elevated in the hippocampus and cortex with exercise (Neeper et al, 1995; Neeper et al, 1996; Gomez-Pinilla et al, 1998). BDNF is transported anterogradely and retrogradely, where it effects gene transcription, morphology, synaptic transmission and neuroprotection in the brain (Cotman &

Berchtold, 2002). Specifically, BDNF was involved in LTP (Patterson et al, 1992), memory (Fischer et al, 1987), synaptic strength changes (Schuman, 1999; Lu & Chow, 1999), progenitor cell survival and differentiation (Ray et al, 1997), and protecting neurons from free radical damage (Spina et al, 1992). In another study, not only was BDNF and TrkB expression increased in the hippocampus after voluntary running, but gene expression decreased with the deprivation of running (Widenfalk et al, 1999). Mice with BDNF deficiency exhibited less synaptic innervation, lower levels of synaptic vesicle proteins, and weakened long-term potentiation (LTP) and learning that are all reversed with BDNF replacement (Korte et al, 1995;1996; Patterson et al, 1996). BDNF binds to TrkB pre-synaptically where it acts on neurotransmitter release, and post-synaptically where it can modify other receptors sensitivity (i.e. increase in NMDA receptor activity) (Black, 1999; Kohara et al, 2001). Furthermore, an increase in BDNF with exercise can modify neuronal and synaptic function via regulating CREB, SYN1, NMDA receptor, synaptophysin (Vaynman et al, 2006), neurotransmitters (Knusel et al, 1992), and CAMKII and MAPK pathways (Vaynman et al, 2003).

Insulin-like growth factor 1 (IGF1) is also increased with exercise. When IGF1 is blocked, so are the exercise induced increases in cfos (Carro et al, 2001) and neuron proliferation (Trejjo et al, 2001) in the brain. Also, without IGF1 exercise no longer improves recall nor increases BDNF levels and synaptic function of downstream proteins (Ding et al, 2006). Moreover, when IGF1 was injected into sedentary animals, the resultant cfos and BDNF expression levels were similar to that of exercised animals (Carro et al, 2001). Increased IGF1 levels reduced risk of health complications such as diabetes, hypertension, cardiovascular disease, and neurodegeneration (Cotman et al, 2007; Colao et al, 2008).

Other molecular factors that increased in level and/or activity due to exercise, and played a role in exercise induced changes in the brain, were 5HT (Blomstrand et al, 1989; Jacobs & Fornal, 1998; Bliss et al, 1983), the NMDA NR2B receptor (Farmer et al, 2004), acetylcholine (Fordyce & Farrar, 1991) and proinflammatory cytokines (Ang & Gomez-Pinilla, 2007).

Exercise Effects in the Spinal Cord

As early as the 1940s, researchers demonstrated that motoneurons exhibit changes in response to a stressor (Hyden, 1947). This information established a basis for the adaptation of the nervous system to exercise. Since that time, others have shown changes in the spinal cord, and also more specifically in motoneurons, in response to exercise.

Reports of changes in gene expression due to exercise are conflicting. This may be due to differing animal models, duration and intensity of activity, and also location of measurements studied. For example, Widenfalk et al (1999) reported no change in RET, p75, NT3, TrkB or BDNF mRNA in alpha motoneurons of the lumbar enlargement after 5 weeks of voluntary running in spontaneously hypertensive rats. However, Ying et al (2003) found an increase in NT3 and TrkC mRNA and protein levels in the dorsal horn after 7 days of voluntary exercise. Furthermore, voluntary exercise increased expression of BDNF, TrkB, SYNI, GAP43, and CREB in the lumbar spinal cord (Gomez-Pinilla et al, 2004). An increase in SNAP25 in motoneuron axons (Kang et al, 1995), and CGRP in the motoneuron soma and sciatic nerve (Gharakhanlou et al, 1999) provide evidence for increased protein synthesis (Gerchman et al, 1975) as well as transport of larger amounts of protein in motoneuron axons with exercise (Dahlstrom et al, 1978). Motoneurons from endurance-trained animals sprouted more after

partial denervation compared to untrained motoneurons (Gardiner et al, 1984), providing further support of increased protein synthesis and axon transport with increased physical activity in an injury model. Additional evidence to support the hypothesis that changes in neuromuscular activity alter gene expression in the spinal cord was an inactivity model where decreasing neuromuscular activity resulted in decreased levels of BDNF, NT3 and SYNI (Gomez-Pinilla et al, 2004). This study also illustrated that the contribution of supraspinal and muscle afferent input modulated BDNF and NT3 expression in the spinal cord. Hence, it is possible that, like the hippocampus, motoneurons can similarly 'learn' physical activity during endurance training (Gardiner et al, 2005).

Electrophysiological studies showed changes in intrinsic motoneuron properties in response to endurance training, providing further support to the hypothesis that motoneurons adapt to physical activity in a 'learning' fashion. For example, after long-term voluntary running there was a hyperpolarized resting membrane potential and voltage threshold, faster action potential rise time, and increased AHP amplitude in slow motoneurons (Beaumont & Gardiner, 2002). Alternatively, a decrease in activity showed motoneuron property changes in the opposite direction that were restored with increased activity (Beaumont et al, 2004). It has not been elucidated which ion conductances direct such changes. Electrophysiological modeling studies suggested an increased sodium and decreased leak conductance (Gardiner et al, 2006). Motoneuron excitability can also be increased by 5HT and NE released in the ventral horn of the spinal cord (Elliott & Wallis, 1992). If exercise effects how motoneurons respond to 5HT in healthy intact animals is presently unknown. However, it was demonstrated that passive limb exercise in a rat model of spinal cord injury upregulated 5HT2A and 7 receptors expression in extensor motoneurons (Chopek et al, 2015). Furthermore, it has been shown that 5HT levels in

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the brain are increased with exercise (Aan het rot et al, 2009; Dunn et al, 1996), and activation of the 5HT1 receptor decreases motor output whereas the 5HT2 receptor facilitates motor output (Schmidt & Jordan, 2000).

It is interesting to note that 7 days of voluntary exercise led to an increase in oligodendrogenesis in the thoracic spinal cord of mice (Krityakiarane et al, 2010). Hence, motoneurons, along with other spinal cord elements, display adaptations to changes in neuromuscular activity, that are most likely preceded by changes in gene expression. For example, using an injury model, electrical stimulation of a nerve after axotomy increased gene expression as evidenced by sprouting (Gordon, 2010). Hence, it is possible that changes in neurotransmitter release from afferents in the spinal cord due to changes in gene expression at the DRG could result in functional change at the synaptic level (i.e. increased synaptic efficacy with increased neuromuscular activity).

Exercise Effects in the Dorsal Root Ganglia

Given all the aforementioned changes to other areas of the nervous system (i.e. CNS) with exercise, it would not be surprising to find changes in the response of sensory neurons (i.e. PNS) to exercise as well.

Exercise performed after spinal cord (SCI) and nerve injury has beneficial effects on activity-dependent plasticity, nerve regeneration, as well as the neuropathic pain response. Keeler et al (2012) showed that passive cycling after SCI resulted in no change in mRNA levels of BDNF, NT3, NT4, GDNF and their receptors in large laser-captured lumbar DRG neurons of the rat. However, changes were noted at the motoneuron and whole spinal cord levels. In another

study investigating SCI, rats exercised on automated wheels for two and seven weeks, five days post-SCI injury, preventing the SCI-induced decrease in GDNF protein levels in the dorsal horn of the spinal cord, as well as in cervical whole DRGs. The attenuation was accompanied by a decrease in tactile allodynia, indicating that exercise prevents the onset of neuropathic pain associated with SCI (Detloff et al, 2014). Additionally, five days of treadmill training after sciatic nerve injury resulted in a decrease in BDNF, NGF and GDNF, and no change in NT3 mRNA levels in whole lumbar DRGs of the rat, compared to injured rats without exercise (Cobianchi et al, 2013). These gene expression results were accompanied by a decreased neuropathic pain response, further supporting the hypoalgesic effect of treadmill training after peripheral nerve lesion. Chen et al (2014) used the skin/muscle incision and retraction surgery as a rat model of inducing postoperative pain. Sedentary animals exhibited prolonged mechanical hypersensitivity, as well as an up-regulation of SubP protein in the DRG. Four weeks of forced treadmill running shortened the recovery time from persistent pain, in addition to reversing the increase of SubP.

Information regarding exercise effects on DRG gene expression in diabetics is limited. In 2013, Groover et al studied mice fed high fat diets to induce pre-diabetes and associated painful neuropathy. Twelve weeks of voluntary wheel exercise improved neuropathic symptoms including normalized: neurotrophin levels, epidermal fiber density, mechanical allodynia and visceral hyperalgesia. Specifically, there was no change in the protein level of GDNF, NGF or BDNF in the DRG in standard diet exercised mice compared to standard diet sedentary controls. However, there was an increase in NGF protein in the DRG, muscle, and skin in high-fat fed sedentary mice, which was significantly normalized in the skin by exercise.

The only study to date supporting the DRG neuron response to exercise in the healthy, non-injured rat is by Molteni et al (2004). They showed that 7 days of voluntary activity increased mRNA levels of BDNF, GAP43, SYNI and NT3 in whole DRG, as well as neurite outgrowth in culture, suggesting exercise increased neuron growth capacity by increasing neurotrophin signalling. This study provides evidence that the DRG is responsive, and can potentially adapt, to increases in activity. To our knowledge, our study is the first to quantify and compare mRNA levels, using qPCR, of small and large laser-captured DRG neurons after exercise in both healthy and diabetic rats.

Exercise-induced Hypoalgesia

Sensory hypoalgesia is a decreased sensitivity to painful stimuli. Exercise analgesia is the increase in pain threshold, or decrease in pain sensitivity, associated with exercise. This phenomenon has been well cited in the literature (Koltyn, 2000). However, mechanisms for this analgesic response are still unclear.

The idea that exercise can alter pain perception was first presented with anecdotal evidence from athletes and dancers who continued strenuous activity and reported no pain despite severe injury. The first study to examine this phenomenon in humans was by Black et al 1979, who found that the pain threshold increased immediately following 40 minutes of running. Hence, the concept of exercise producing a decreased pain response is not a new idea. However, the basic mechanisms that contribute to this response are still largely unknown. One popular hypothesis, is that exercise causes an increase in opioid levels, which results in antinociception during exercise, and even after exercise has ceased (Shyu et al, 1982; Hoffmann et al, 1990;

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Smith & Yancey, 2003; Smith & Lyle, 2006). Thermal discriminability and ischaemic pain was reduced in long distance runners along with increased plasma beta endorphin, insulin receptor, ACTH, GH, and PRL levels (Janal et al, 1984). However, only portions of the hypoalgesic response and mood elevation were reversed by naloxone (opioid antagonist) suggesting the endogenous opioid system as only a partial contributor to the response Therefore, it is evident there are opioid and non-opioid analgesia mechanisms, with the non-opioid mechanisms being largely unexplored.

Whether there is an exercise-induced hypoalgesic response or not, seems to depend on the intensity and type of exercise and inter-individual variability (Koltyn, 2002; Hoffmann et al, 2004). The type of pain (i.e. pressure, thermal, electrical, etc.) and the anatomical location (i.e. hand vs. foot; and in relation to the stressed muscle) being tested, also determines the analgesic response to exercise, and accounts for variability of the pain response. Endurance training resulted in a higher noxious cold threshold (Janal et al, 1994) and a lower discriminability to low intensity radiant heat pain (Fuller & Robinson, 1993). A decrease in pressure pain has also been found after isometric fatiguing contractions (Hoeger Bement et al, 2009), in fast ultramarathon runners (Hoffmann et al, 2007), with stepping at a submaximal workload (Gurevich et al, 1994) and during self-selected exercise in a gym (Bartholomew et al, 1996). Pressure pain induced increases in motor-evoked potential (MEP) amplitude were attenuated following exercise suggesting a decrease in net corticomotor excitability corresponding to a decrease in pain (Hoeger Bement et al, 2009).

Previously, research investigating human exercise-related modulation of pain involved cycling exercise and decreased pain response to electrical (Kemppainen et al, 1985), temperature (Sternberg et al, 1998), and pressure (Koltyn et al, 1996) stimuli. In animals, the main exercise

stimulus was swimming, using thermal (Christie et al, 1981) and electrical (Bodner et al, 1978) pain stimuli to show increased pain thresholds. The majority of mechanistic studies utilized naloxone to determine the opioid response to exercise in rodents (Tierney et al, 1991; Bodnar et al, 1978; Willow et al, 1980; O'Connor & Chipkin, 1984) and humans (Haier et al, 1981; Janal et al, 1984; Droste et al, 1991 & 1988; Olausson et al, 1986). The results concluded that increased pain thresholds and tolerances as well as lower pain ratings occur with exercise, and occur consistently more with high intensity exercise (Koltyn, 2000). The results regarding how exercise-induced changes in body temperature related to heat or cold pain thresholds were unclear.

More recently, animal studies investigating exercise analgesia have incorporated voluntary running exercise (Mathes & Kanarek, 2006). However, a highly popular hypothesis still being studied is that exercise causes an increase in opioid levels which results in anti-nociception, even though it has been shown that naloxone is not effective in attenuating the pain response in all instances (Koltyn et al, 2014). For this reason, we chose to include the δ- and μ-opioid receptors to our study. Furthermore, since the mechanistic role of genes in exercise analgesia, other than opioids, has not been extensively explored or examined, we studied several other genes that are known to be involved in the pain response (please refer to section 5 of introduction).

Effects of exercise in a pain model. Chronic pain is a condition that costs Canadians billions of dollars annually (www.canadianpainsociety.ca) and imposes on the physical and emotional well-being of 1 in 5 Canadians every day (Moulin et al, 2002; Choiniere & Dion, 2010; Schopflocher et al, 2011). Pain contributes to ongoing suffering, disability, and poor quality of health (including physical, mental and emotional), among other harmful effects

(Liebeskind, 1991). Furthermore, untreated pain experienced in early childhood can lead to persistent changes in sensory processing (Lynch, 2011). For example, as high as 30% of children experience pain which can interfere with daily functioning, mental health, and school performance (Gauntlett-Gilbert & Eccleston, 2007) and can increase the risk of chronic pain later in life (Ramage-Morin & Gilmore, 2010; Jones et al, 2009; Stanford et al, 2008). Chronic pain can affect various aspects of an individual's life, evidenced by job loss and the negative impact on relationships with family and friends (Lynch, 2011). Moreover, only 54% of Canadians diagnosed with chronic pain have a treatment plan, and 45% believe there are no treatment options (www.canadianpainsociety.ca).

Exercise leads to a decreased pain response in several pain conditions. Exercise reduced neuropathic pain associated with SCI (Hutchinson et al, 2004), and allodynia after peripheral nerve injury improving functional recovery (Cobianchi et al, 2010). Exercise also reversed thermal and tactile hypersensitivity caused by spinal nerve ligation (Stagg et al, 2011). This study also found that increased beta-endorphin and met-enkephalin levels in the PAG and RVM caused by exercise, was required to reverse neuropathic pain (Stagg et al, 2011). Chronic muscle pain was also reversed by exercise, and this effect was inhibited by naloxone (Bement & Sluka, 2005). Inflammatory pain was diminished by swimming (Kuphal et al, 2007). Exercise resulted in functional improvement and decreased chronic pain in patients with osteoporosis (Malmros et al, 1998), fibromyalgia (McCain et al, 1988; Gowans & deHueck, 2004), low back pain (Hayden et al, 2005; Chatzitheodorou et al, 2007; Hoffmann et al, 2005), and cancer-treatment related pain (Robb et al, 2006). In a mouse model of chronic pain, exercise decreased cutaneous and deep tissue hyperalgesia, and stimulated NT3 synthesis in the gastrocnemius but not the soleus

muscle (Sharma et al, 2010). This study was the first to indicate a molecular basis of how exercise acts to reduce muscular pain.

Hence, the nervous system response to exercise, and exercise analgesia mechanisms, may vary based on health status (i.e. healthy vs. pain model).

3. DRG Neurons are Altered with Diabetes

Diabetes

Diabetes is a chronic disease where blood glucose levels are elevated. There are 347 million people worldwide that currently have diabetes (WHO, 2014). Approximately 3.4 million people die each year from the disease (WHO, 2011). There are four main types of diabetes: type 1, type 2, gestational, and secondary. Type 1 diabetes occurs when the insulin-producing beta cells of the pancreas are destroyed leading to insulin insufficiency, whereas type 2 diabetes occurs when cells are resistant to insulin. Gestational diabetes occurs when insulin receptors do not function properly usually involving insulin resistance during pregnancy. Secondary diabetes occurs as a complication of some other disease, and can either result in insulin resistance or insulin deficiency. All of these types of diabetes lead to elevated blood glucose levels (i.e. hyperglycemia). Study 2 of this thesis incorporates the streptozotocin (STZ) rat model to examine type 1 diabetes.

STZ was discovered in 1959 as an antibacterial antibiotic (Vavra et al, 1959). In 1963, Rakieten et al showed that STZ produced a diabetogenic action, via pancreatic beta-cell necrosis (Junod et al, 1967) resulting in hyperglycemia (Rerup et al, 1970) and type 1 diabetes (Rossini et al, 1977). STZ is selectively toxic to beta-cells since they have high levels of the Glut2 glucose

transporter, and STZ is transported into the cell specifically by Glut2 (Schnedl et al, 1994; Wang et al, 1998). STZ destroys the cells by damaging the DNA, which activates poly ADP-ribosylation and further downstream pathways that result in the generation of reactive oxygen species (Szkudelski et al, 2001; Lenzen et al, 2008).

Diabetic Peripheral Neuropathy

Diabetic neuropathy (DPN) occurs in approximately 60-70% of patients with diabetes, and is most commonly the distal symmetrical form (for a review on pathogenesis and management of DPN please see Farmer et al, 2012). DPN consists of damage to peripheral nerves due to complications from diabetes. DPN can present as painful (for review please see Lee-Kubli & Calcutt, 2014) or more commonly as insensate. DPN with associated neuropathic pain results in symptoms such as a tingling or burning sensations and increased sensitivity to touch (Schreiber et al, 2015). Alternatively, DPN associated with decreased pain transmission results in symptoms such as numbness and weakness in the hands and feet (Spallone & Greco, 2013). Study 2 focused on the latter type of DPN, with the behavioral evidence of thermal hypoalgesia. Since the mechanisms of DPN are not fully understood, there is minimal treatment available, and the condition can progress to ulcers and even amputation of the limb affected (Turns, 2015).

Insensate neuropathy. Some animals/humans with diabetes experience neuropathic pain while others exhibit a decreased pain response (i.e. hypoalgesia), even though they both have DPN (ie. dying back neuropathy and sensory loss). One hypothesis is that in some patients the dying back progresses so quickly the development of pain is by-passed. In animal models, rat or

mouse pain develops early, especially allodynia, to be later replaced by sensory loss. It is not known why this happens, but probably physiological responses are altered in diabetes. For instance, these altered responses occur early and primarily direct pain, whereas the structural deficits leading to hypoalgesia take longer to develop and thus may displace pain (Fernyhough, 2015). Hence, we examined DPN and exercise to investigate the thermal hypoalgesia response.

Insensate DPN is a dying back neuropathy, including structural damage to nerve endings, leading to sensory loss. There are various theories regarding mechanisms including: 1) polyol pathway flux, oxidative stress and advanced glycation end-products (AGEs) (Hinder et al, 2012; Ahmed, 2005; Tomlinson, 1992); 2) loss of neurotrophic support (Fernyhough et al, 1998); and 3) mitochondrial dysfunction and calcium dyshomeostasis (Fernyhough & Calcutt, 2010; Fernyhough et al, 2010).

There is a decrease in Aβ-fibers in the skin of animals with insensate neuropathy, causing functional deficits in sensory signalling (Lennertz et al, 2011). There is also small fiber loss in the skin associated with the nerve endings dying back, similar to that in humans (Johnson et al, 2008). This cutaneous fiber loss is the main cause of insensate diabetic neuropathy. It is common for type 1 diabetic patients to experience tactile allodynia followed by hypoalgesia as the neuropathy progresses (Yagihasi, 1995; Calcutt, 2002). In STZ rats, tactile allodynia is evident by both the light touch of a von frey filament and a light paw stroke with a cotton bud resulting in a withdrawal response, just days after diabetic onset (Calcutt et al, 1996; Field et al, 1999). STZ rats begin with a slowing of MNCV and SNCV at 1-2 months of becoming diabetic (Arezzo & Zotova, 2002), preceding hypoalgesia.

Next, behavioural testing demonstrates thermal hypoalgesia at 3-4 months, followed by a decrease in epidermal nerve fibers (ENF) (i.e. consists primarily of heat-sensitive C–fibers) at 6

months or more. Furthermore, a decrease in function of certain sensory nerves (i.e. thermal hypoalgesia) occurs 2 months earlier than cutaneous nerve fiber loss (Chen et al, 2005). There is a reduction in Fos positive cells in the dorsal horn of the spinal cord that corresponds with peripheral axon loss and resultant sensory loss (Johnson et al, 2007). Beiswenger et al (2008) describes this similar phenomenon in STZ mice with hypoalgesia evident after 2 weeks of diabetes, and a decrease in ENF density after 4 weeks, indicating compromised epidermal nociceptor function, before the loss of peripheral terminals.

Furthermore, IGF-1 treatment can reverse the decrease in peripheral sensory transmission associated with DPN, but not the changes to the central somatosensory system (Piriz et al, 2009). Hence, there are not only peripheral deficits associated with DPN, but functional deficits in activation at the brain and spinal cord levels as well.

DPN progression. Why neuropathy ensues in over half of diabetic patients is not known. The pathogenesis is multifactorial and several mechanisms have been proposed (see Albers & Pop-Busui, 2014 for review). There are some variations between the STZ rat model we used for this study and diabetic progression in humans, but both lead to permanent hyperglycemia. The main difference between human and rat DPN, is that humans can experience peripheral large diameter nerve loss (though less common than small), whereas this is not evident in the STZ rat model (although they do exhibit shrinkage of myelinated axons). However, there is abnormal muscle spindle innervation noted in mice, replicating large fiber neuropathy, that may contribute to abnormal gait (ie. sensorimotor function) (Muller et al, 2008; For review of diabetic motor neuropathy see Wilson & Wright, 2014).

Pathologies associated with peripheral neuropathy are nerve fiber loss, axon atrophy and demyelination (Dyck & Giannini, 1996). Length-dependent diabetic polyneuropathy is the most

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common form of diabetic neuropathy. The longest nerve fibers show damage first, and associated signs and symptoms are apparent in the patient (Said, 2007). This type of diabetic neuropathy is a progressive condition where small fibers seem to degrade first followed by the larger fibers (Umapathi et al, 2007; Quattrini et al, 2007; Loseth et al, 2008). The number of small peripheral nerve fibers is much greater than the number of large ones (Said et al, 2008; Malik et al, 2005). Small fiber length-dependent polyneuropathy involving unmyelinated sensory loss is the more common form of diabetic neuropathy compared to the large-fiber form involving myelinated fiber loss (Said, 2007). Small diameter fibers that innervate the skin transmit both nociceptive and thermal sensations (Harper & Lawson, 1985). Furthermore, peptidergic fibers are lost prior to, and to a greater extent than, nonpeptidergic fibers, stressing the fibers' role in early cutaneous insensitivity (Johnson et al, 2008). Study 2 will examine both the small and large sensory neuron cell bodies, generally associated with their small and large fibers respectively (Harper & Lawson, 1985).

In summary, the progression of diabetic neuropathy starts with metabolic disturbance, followed by neurologic complications, and finally pathologic changes to nerve fibers (Thomas & Tomlinson, 1993).

Effects of Exercise on Diabetic Peripheral Neuropathy

We used the STZ rat model to look at the effects of exercise on diabetics. Hyperglycemia can lead to serious organ damage as well as nerve and blood vessel damage. Cotman et al (2007) suggested that exercise reduces peripheral risk factors for diabetes such as hyperglycemia and others involving systemic inflammation.

Exercise is a recognized form of therapy for diabetics and long-term aerobic training can actually delay and/or prevent the development of both motor and sensory DPN, as first shown by Balducci et al, 2006. Forced exercise also restored voltage gated calcium channel function in small DRG neurons to delay the onset of neuropathic pain (Shankarappa et al, 2011). Furthermore, in a rat neuropathic pain model, regular exercise reversed sensory hypersensitivity (Stagg et al, 2011).

Exercise is effective in the recovery of motor function in rats with DPN (Malysz et al, 2010) as evidenced by: restored compound muscle action potential (CMAP) amplitude and CMAP latency (Selagzi et al, 2008), increased tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta (do Nascimento et al., 2010), and improved motor and sensory nerve conduction velocity (Van Meeteren et al, 1996; Fisher et al, 2007; Hung et al, 2009). Exercise also resulted in enhanced intraepidermal nerve fiber (iENF) density in humans, resulting in cutaneous reinnervation and reduced pain in pre-diabetic humans (Smith et al, 2006). Other beneficial effects of exercise in diabetics were improved neuropathic symptoms and cutaneous nerve fiber branching (Kluding et al, 2012), and increased size of A cells from L5 DRGs (do Nascimento et al, 2010).

Exercise also improved cardiorespiratory fitness and decreased risk of complications associated with type 1 diabetes and mortality (Briscoe et al, 2007; Rachmiel et al, 2007). Exercise increased circulating IGF1 levels (Carro et al., 2001) which could contribute to a decreased risk of diabetes. Other benefits of exercise include: increased cardiorespiratory fitness (Nielsen et al, 2006), reduced morbidity and mortality (Moy et al, 1993), increased vigor (CDA, 2008), decreased insulin resistance (CDA, 2008), and improved lipid profile and maintenance of weight loss (Wing et al, 2011).

One of the risks associated with an insulin-dependent patient partaking in aerobic exercise is insulin and glycemic imbalance. This risk can be reduced by appropriate glucose supplementation and/or timing of insulin injection assessed on an individual basis (Francescato et al, 2011; Bracken et al, 2011; Gulve, 2008). Glucose regulation is a treatment that, when enforced early, can delay diabetes and even delay and reverse DPN (Zilliox & Russell, 2011). In fact, a combined aerobic and resistance training routine can actually lower daily insulin requirements and improve physical fitness, general health, and well-being (D'hooge et al, 2011). Therefore, the type of aerobic exercise affects the outcome for patients. Furthermore, the intensity of exercise performed also determines the glucose response in diabetics. Moderate intensity exercise resulted in hypoglycemia, intermittent high-intensity exercise made patients hyperglycemic, while a combination of the two still produced decreased glucose levels and protected against nocturnal hypoglycemia (Iscoe & Riddell, 2011).

Hence, exercise is a good tool to reduce the risk of developing diabetes, and also to treat diabetes. Physical activity, in addition to good glycemic control, results in less neuropathy (Loprinzi et al, 2014). However, less has been studied regarding the effect of exercise on insensate diabetic neuropathy in particular, and what the molecular mechanisms of change are. Therefore, we studied STZ rats with DPN in order to show how exercise could affect the onset of thermal hypoalgesia, and the corresponding small and large DRG gene expression changes.

4. Purpose of and Rationale for the Current Study

This thesis comprises an investigation into the differences in gene expression of small and large DRG neurons, in healthy and diabetic rats, in response to exercise. Based on the results, underlying mechanisms were suggested for DPN and exercise analgesia. The central

hypotheses are that: 1) large and small DRG neurons vary in their gene expression of genes not studied previously in DRG subtypes using LCM, in healthy sedentary, healthy exercised, diabetic sedentary and diabetic exercised rats 2) changes in DRG gene expression in response to acute and chronic exercise differ 3) voluntary wheel exercise causes changes in gene expression in small and large DRG neurons that differ from the changes resulting from treadmill exercise 4) exercise delays the onset of thermal hypoalgesia associated with DPN.

The testing of these hypotheses involves a combination of exercise protocols, molecular biology techniques as well as behavioral testing in the rat. The availability of LCM equipment and both healthy and STZ rats, allowed us to separate small and large DRG neurons in both normal and diabetic rats in order to address these hypotheses.

As described above, there is only sparse information regarding the effect of exercise on healthy rat DRG gene expression. Therefore, we used the reliable qPCR method of mRNA quantification as a measurement of the effects of exercise, mainly because it is sensitive to the small amounts of RNA extracted from the DRG neuron single cells that were pooled together. Moreover, the novel approach of isolating small and large DRG neurons via LCM has not been used to investigate sensory signalling changes associated with DPN or in healthy animals with exercise. Due to the discrepancy of previously reported gene detection in the DRG of healthy sedentary rats, our study will also aid in substantiating these findings (Please see section 5 of introduction for more details). The gene expression results, in terms of exercise and diabetes, produce the first information of its kind of which we are aware.

Study 1

It was previously demonstrated that the expression of BDNF, NT3, Syn1 and GAP-43 were up-regulated in healthy, non-injured rat DRGs after 7 days of voluntary exercise (Molteni et al., 2004). We wished to extend these observations to include forced treadmill exercise, add expression of other physiologically relevant genes (please refer to section 5 of introduction), add a longer period of exercise, and determine the mRNA levels of each gene in small (<=30um) and large (>=40um) DRG neurons.

It is evident that whole DRG are responsive to acute increases in neuromuscular activity in healthy rats (Molteni et al, 2004), and rats with SCI (Detloff et al, 2014) and sciatic nerve injury (Cobianchi et al, 2013). DRG gene expression changes are also noted after chronic exercise in rats with SCI (Detloff et al, 2014), pain (Chen et al, 2014) and DPN (Groover et al, 2013). However, such chronic changes have not been studied in healthy rats. Furthermore, neither acute nor chronic exercise effects on small and large DRG neurons in the healthy rat have been studied. Therefore, we studied chronic exercise in healthy rats to determine if DRG neuron mRNA changes occur, and if so, how these changes influence small and large DRG neuron gene expression.

Neurons that have a change in activity exhibit adaptations that are most likely preceded by changes in gene expression. For example, three days of treadmill exercise prior to induced heart injury resulted in no change in synthesis or release of CGRP, whereas 3 weeks of exercise resulted in increased CGRP gene expression in the DRG as well as release in the blood and heart of rats, hence, having a cardio-protective effect (Sun & Pan, 2014). This study also demonstrates differing effects of acute verses chronic exercise. Keeler et al (2012) showed increased GDNF family receptor α1 (GFRa1) mRNA levels in large DRG neurons of rats subjected to SCI followed by 31 days of exercise, which may correspond to enhanced functional recovery.

However, ten days of exercise post-injury did not show any significant change in mRNA levels. In the healthy rat, acute exercise caused an increase in whole DRG mRNA levels of genes related to the resultant increased neurite outgrowth (Molteni et al, 2004).

Based on this information, the following hypothesis was generated:

__Hypothesis 1.1:__ Gene expression of small and large DRG neurons shows greater differences with 17 weeks compared to 1 week of forced treadmill exercise

The difference between the effects of treadmill verses wheel running exercise have not been extensively studied. Treadmill exercise is focused on higher intensity and greater total distance, whereas voluntary wheel exercise is associated with lower intensity but greater time per day. Thus, some genes may be more sensitive to increased intensity of training, while others more affected by time spent training. Moreover, treadmill training can be considered forced behavior which may enhance stress (Conner et al, 2014, Alaei et al, 2007, Ploughman et al, 2005, Yanagita et al, 2007) and lead to anxiety (McDevitt & Neumaier, 2011), whereas running on a wheel is voluntary. However, others believe treadmill running is actually more similar to how humans exercise (Di Loreto et al, 2014) compared to voluntary exercise. Treadmill trained animals can adapt to stress, evidenced by return of stress levels to normal after daily training (Grissom & Bhatnagar, 2009). Whether small and large DRG neurons respond differently to voluntary wheel verses forced treadmill exercise has not been previously examined. However, effects of treadmill (Chen et al, 2014; Cobianchi et al, 2013; Sun & Pan, 2014) and voluntary wheel (Detloff et al, 2014; Groover et al, 2013) exercise on whole DRG in non-healthy rodents have been investigated separately, but not in comparison. Likewise, whole DRG gene expression

was studied in healthy rats following acute voluntary wheel training (Molteni et al, 2004), but not treadmill training.

Based on this information, the following hypothesis was generated:

Hypothesis 1.2: *Gene expression of small and large DRG cells of chronic treadmill exercised animals is different from that of voluntary wheel trained animals.*

Objectives

I.   To develop a protocol to visualize and isolate small and large DRG neurons while maintaining the integrity and maximizing the concentration of the sample RNA

II.   To establish qPCR parameters for the purpose of our study including determining the genes of interest

III.   To identify the relative changes in mRNA for each gene between small and large DRG neurons following treadmill and voluntary wheel training.

IV.   To evaluate if long-term trained small and large DRG neurons exhibit the same differences in gene expression as short-term trained neurons

Study 2

The first study showing the depletion of cutaneous nerves and neuropeptides in patients with diabetic neuropathy was released in 1989 (Levy et al., 1989). Abnormal innervation was examined using the immunocytochemical study of skin biopsies. Later, in 1996, Kennedy et al.

further quantified the epidermal nerves in patients with diabetic neuropathy via a neuron tracing system, revealing a decrease in the number and length of epidermal nerve fibers.

Christianson et al 2003 were the first group to show iENF loss in skin in a mouse model of diabetes. The Wright laboratory proceeded to investigate the effect of exercise on diabetic neuropathy following these key findings. Namely, Kluding et al 2012 completed the first study examining the effects of 10-week aerobic and resistance exercise programs on nerve function in patients with DPN. Exercise resulted in decreased pain and neuropathic symptoms, and improved iENF branching of neuropathic and cutaneous nerve fibers. Subsequently, Groover et al 2013 used mice fed high fat diets to induce pre-diabetes and associated painful neuropathy. Twelve weeks of voluntary wheel exercise improved neuropathic symptoms including normalized mechanical allodynia and visceral hyperalgesia, neurotrophin levels, and epidermal fiber density. Furthermore, it was shown in human studies that long-term aerobic exercise can delay and even prevent the onset of DPN (Balducci et al, 2006), and forced exercise has been shown to delay the onset of neuropathic pain sometimes associated with DPN (Shankarappa et al, 2011). Although our study does not involve neuropathic pain or forced exercise, but rather thermal hypoalgesia and voluntary wheel exercise, these studies provide evidence that DPN is affected by exercise.

In our study, after becoming diabetic, some STZ rats were sedentary while others began to exercise on a voluntary wheel. Throughout the protocol, behavioral testing was used to investigate nerve function. Specifically, the Hargreaves test (Hargreaves et al, 1988) displays the animal's response to heat by selectively activating C fibers, and determines thermal hypoalgesia which is indicative of DPN. A focused, radiant heat light source elicited a paw withdrawal response. An increased paw withdrawal response latency indicates C fiber transmission dysfunction, thermal hypoalgesia, and even cutaneous fiber loss, which are all signs of DPN.

Therefore, whether or not withdrawal latencies are altered can determine if exercise causes a delay in the onset of thermal hypoalgesia (i.e. functional change). If so, exercise could be used as a form of DPN prevention and/or treatment.

Therefore, our study addressed the effect of voluntary chronic exercise on thermal hypoalgesia in STZ rats, and whether or not gene expression changes at small and large DRG cells provide mechanistic insight into DPN progression.

Based on this information, the following hypothesis was generated:

Hypothesis 2.1: *Diabetic rats that exercise will experience a delay in the onset of thermal hypoalgesia.*

Hypothesis 2.2: *There will be differences in small and large DRG gene expression in response to exercise, providing evidence of molecular mechanisms to support hypothesis 2.1.*

Objectives

I.   To determine if diabetic rats that exercise experience a delay in onset, or prevention of thermal hypoalgesia.

II.   To determine if functionally significant gene expression in small and large DRG neurons of diabetic animals is modified, and how this regulation is affected by exercise.

5. Genes of Interest in the DRG

Changes in gene expression in DRG neurons may have an effect at the DRG soma, and the peripheral and central terminals, depending on the particular gene and its function. For

example, an alteration in gene expression could result in the altered protein synthesis, which could impact DRG neuron excitability, synaptic efficacy at central projections, as well as peripheral signaling. The genes of interest can be classified as: ion channels, receptors, genes related to growth, synaptic vesicles and pain, as well as neuropeptides.

Sodium (Na) Channels

Primary sensory neurons "encode their messages in the form of a series of action potentials whose depolarizing upstroke is produced by sodium channels" (Qi et al, 2011). Catterall et al (2005) summarized voltage-gated sodium channels (Na_v) as "critical for initiation and propagation of action potentials and for the regulation of neuron excitability". There are nine alpha sub-units and four beta sub-units. TTX-sensitive (TTX-S) channels display fast Na currents, while TTX-resistant (TTX-R) channels display slow Na currents. The TTX-S Na_v channels are 1.1, 1.2, 1.6 and 1.7. The TTX-R Na_v channels are 1.8 and 1.9.

Figure 3: Array of voltage-gated sodium channels (Na_v) alpha subunits

Na_v isoform	TTX sensitivity
Na_v1.1	TTX-S
Na_v1.2	TTX-S
Na_v1.3	TTX-S
Na_v1.4	TTX-S
Na_v1.5	TTX-R
Na_v1.6	TTX-S
Na_v1.7	TTX-S
Na_v1.8	TTX-R
Na_v1.9	TTX-R

(Adapted from Theile & Cummins, 2011)

The voltage-gated sodium channels expressed in rat DRG are: the TTX-S Na_v1.1, 1.6 and 1.7 and the TTX-R Na_v1.8 and 1.9 (Rush et al, 2007). The TTX-S Na_v1.2 and 1.3 channels are mainly active in embryonic tissue. However, they can be activated in adult sensory neurons under certain circumstances. For example, in axotomized small DRG neurons which exhibit a more hyperpolarized threshold for overshooting action potential, Na_v1.8, which largely produces the inward current associated with action potential upstroke during repetitive firing, was downregulated; and Na_v1.3, which was previously silent, became the major contributor to the upstroke (Rush et al, 2007). Study 1 included Na_v1.2, which is known to emerge in MS damaged neurons (Craner et al, 2004), and is associated with myelinated axons, along with Na_v1.3, to determine if the channels are active and regulated with exercise.

Ho & O'Leary (2011) used cell culture to separate small and large rat DRG neurons. Using single cell RT-PCR they found Na_v channels 1.1, 1.2, and 1.6-1.9 present in small neurons associated with thin, unmyelinated axons that mainly transmit thermoreception and pain. They also found Na_v channels 1.1, and 1.6-1.8 present in large neurons associated with thick, myelinated axons that mainly transmit low-threshold non-noxious stimuli. Na_v1.7-1.9 were predominately expressed in small neurons, and Na_v1.8 was only expressed in a sub-group of large neurons. Furthermore, studies of kinetics revealed the TTX-R slow current "activated and inactivated over a depolarized range of voltages", whereas the TTX-S fast current showed "rapid kinetics and gated over hyperpolarized voltages".

Another study using cell culture of small rat DRG neurons only, also found the highest levels of Na_v channel mRNA for subunits 1.7-1.9 (Theriault & Chahine, 2014). They also described three sub-groups of small DRG neurons based on the Na_v channels present. For instance, group one consisted of channels 1.8 and 1.9, group two contained channels 1.7-1.9, and

group three expressed 1.7 only. All three sub-groups differed in their action potential properties, however, they exhibited the same firing frequencies and activation thresholds.

$Na_v1.6$ is mainly found in A-type fibers (Wittmack et al, 2005), and is preferentially expressed by TrkC neurons (Fukuoka et al, 2008). Its role in action potential generation was to maintain high frequency firing (Rush et al, 2007). $Na_v1.7$ produced a ramp current leading to amplification of small inputs (Rush et al, 2007) and played a role in acute and chronic pain (Nassar et al, 2004). $Na_v1.7$ was increased in neuropathic pain models (Cummins et al, 2004; Black et al, 2004). Interestingly, the amplification by $Na_v1.7$ to bring membrane potential to threshold, activated $Na_v1.8$ (Rush et al, 2007; Renganathan et al, 2001). $Na_v1.8$ was found mainly in small DRG neurons, was responsive to NGF (Fjell et al, 1999) and 5HT (Akopian et al, 1999), and was involved in pain (Akopian et al, 1999) and neuropathy (Dong et al, 2007; Jarvis et al, 2007; Lai et al, 2002). $Na_v1.9$ was mainly found in C-fiber nociceptors (Fukuoka & Noguchi, 2011) that were IB4 positive (Fang et al, 2006) and was involved in setting the resting membrane potential (RMP), amplifying inputs and maintaining activation of Nav1.8 (Rush et al, 2007). $Na_v1.9$ was upregulated by 5HT (Kerr et al, 2001), associated with pain (Priest et al, 2005) and was also expressed and functioned pre-synaptically in the spinal cord by regulating neurotransmitter release at the primary sensory synapse (Medvedeva et al, 2009).

Sodium channel activation is directly implicated in diabetic neuropathy (Hong & Wiley, 2006; Cherian et al, 1996). $Na_v1.7$ in particular has been implicated in both pain (Cummins et al, 1998; Waxman & Hains, 2006; Dib-Hajj et al, 2013) and diabetic peripheral neuropathy (Craner et al, 2002; Hong et al, 2004; Galloway & Chattopadhyay, 2013). Hence, Na_v 1.3 and 1.6-1.9 modulate nociceptive responses (Laird et al, 2002; Dib-Hajj et al, 2009; Xie et al, 2013; Garrison et al, 2014), including painful neuropathy (Hoeijmakers et al, 2014).

In summary, all of the Na_v channels noted above have different roles to play in electrogenesis within DRG neurons. The particular Na_v channels present in a DRG neuron determine its electrophysiological properties (along with other types of channels contributing to the firing pattern). Thus, small and large neurons differ in excitability, with differing depolarizing and gating properties. Therefore, differences in gene expression of any of these Na_v channels could result in changes to excitability of small and large DRG neurons.

Potassium (K) Channels

K channels were included in study 1 for exploratory purposes, since they are found, and play a role, in virtually all CNS neurons. In addition, K channels are involved in nociceptive and diabetic neuropathic pain processing.

K channels are subdivided into four main groups: 1) Voltage-gated (K_v) 2) Ca2+ activated (K_{Ca}/ SK) 3) Two-pore (K_2P) 4) Inwardly rectifying (K_{IR}). The K channels investigated in study 1 were $K_v1.1$ and 1.2, and $K_{Ca}2.2$ and 2.3. K_v channels have 12 families (K_v1-12) (Gutman et al, 2003). K_{Ca} channels are subdivided both based on their conductance (BK-big; IK-intermediate; SK-slow), with 5 α-subunit members (1.1(BK), 2.1-2.3(SK), & 3.1(IK)), and another subfamily not based on their conductance of Ca but rather on intracellular Na+ and Cl- (4.1, 4.2 & 5.1) (Wei et al, 2005).

$K_v1.1$ and 1.2 are delayed rectifier channels that maintain membrane potential and modulate electrical excitability in neurons (Gutman et al, 2005). $K_v1.1$ and 1.2 mRNAs were abundant in lumbar DRGs (Yang et al, 2004; Kim et al, 2002), and predominantly found in large DRG neurons (Rasband et al, 2001; Vydyanathan et al, 2005). However, $K_v1.1$ channels were

also expressed in high-threshold C-mechano-nociceptors and Aβ-mechanoreceptors, thus playing a role in mechanical perception for both touch and pain (Hao et al, 2013). There was a down-regulation of both K_v1.1 and 1.2 mRNA in rat whole DRG in a chronic pain model of sciatic nerve transection (Yang et al, 2004) and chronic constriction injury (Kim et al, 2002). STZ rats with DPN did not display a down-regulation of either K_v1.1 or 1.2 in whole DRG (Cao et al, 2010). However, in peripheral nerve fiber of both rats and humans with DPN, K_v1.2 was decreased, and this decrease was associated with hyperexcitability of the nerve (Zenker et al, 2012). Contrastingly, there was no change in K_v1.1 or 1.2 nerve protein levels in diabetic rats (Brown et al, 2001).

SK channels play a key role in regulating the AHP in electrically excitable cells (Faber & Sah, 2007). SK2 and 3 channels are similar in both current properties and physiological function (Wei et al, 2005). Sarantopoulos et al (2007) displayed SK currents in both small and medium DRG neurons. The total current, as well as the SK current fraction, were decreased after axotomy and subsequent hyperalgesia.

K_v7 has 3 alpha-subunits (7.2, 7.3 and 7.5) expressed in the peripheral somatosensory system (Du & Gamper, 2013), also known as M channels (K_M). These channels regulate neuronal excitability (Brown, 1988; Marrion, 1997). In particular, this low-threshold channel, when depolarized, displayed a very slow, non-inactivating outward M-current (Huang & Trussell, 2011), that acts to "clamp" the membrane potential, thereby disallowing repetitive action potential firing. Alternatively, activation of muscarinic receptors inhibited the M-current, which allowed sustained action potential firing (Delmas & Brown, 2005). Hence, M channels are important in regulating both action potential and resting membrane potential thresholds in DRGs. There is conflict regarding the exact subunit composition of these channels in the DRG.

However, it is clear they are present and functional in the DRG, contributing to the sustained, delayed-rectifier current (Passmore et al, 2003; Rose et al, 2011; King & Scherer, 2012; Zheng et al, 2013), which is suggested to play a role in neuropathic pain (Du & Gamper, 2013).

In summary, there is a downregulation of K+ channels during chronic pain associated with nerve damage, including DPN (Du & Gamper, 2013). To our knowledge, there has not been any other investigations into the effect of exercise on K channel levels in the DRG.

Transient Receptor Potential (TRP) Channel

TRP channels are a large group of ion channels consisting of 6 subfamilies. The subfamily vanilloid comprises 6 members including TRPV1 (Clapham et al, 2005). TRP channels role in nociception is discussed above in the pain overview section. TRPV1 in particular is involved in noxious heat stimuli transduction at temperatures around 43 degrees Celsius (Caterina et al, 1997; Voets et al, 2004). The receptor can also be activated on nociceptive sensory neurons by capsaicin, which induces a burning pain sensation (Caterina et al, 1997). During inflammation, TRPV1 activation is essential for sensitization to noxious thermal stimuli leading to thermal hyperalgesia (Davis et al, 2000; Catterina et al, 2000).

STZ mice with DPN exhibit a down-regulation of the TRPV1 channel (i.e. thermal hypoalgesia) in DRG neurons as a result of insulin deficiency (Pabbidi et al, 2008). Furthermore, opening of the TRPV1 channel in STZ rats activates caspase and calpain stress pathways in large DRG neurons (Hong et al, 2008).

The effect exercise on TRPV1 expression in the DRG is not known.

Gamma-aminobutyric Acid (GABA) Receptors

GABA$_A$ receptors at inhibitory synapses both reduced nociceptive input via presynaptic inhibition (Kullmann et al, 2005 & 2012) and exaggerated pain and neurogenic inflammation via peripheral afferent depolarization triggering action potentials (Willis, 1999). For instance, when the α2 receptor was deleted from nociceptive primary afferents, diazepam (DZP) sensitivity of GABAergic membrane currents in nociceptive DRG neurons, GABA$_A$ receptor mediated presynaptic inhibition and primary afferent depolarization were reduced. These changes attenuated the anti-hyperalgesic effect of normally acting spinal DZP (Witschi et al, 2011).

Presynaptic GABA$_B$ receptors are involved in the regulation of GABA, glycine and glutamate release in the dorsal horn of the spinal cord (Wang et al, 2006; Li et al, 2002). The GABA$_B$ receptor agonist, baclofen, had an anti-nociceptive effect on both control rats and rats with chronic pain (Smith et al, 1994). Furthermore, in rats with STZ-induced diabetic neuropathic pain, there was a decrease in the activity of the GABAergic inhibitory system, evident by the reduction in the anti-nociceptive effect of baclofen (Malcangio & Tomlinson, 1998) as well as the reduced function of presynaptic GABA$_B$ receptors at primary afferent terminals in the dorsal horn (Wang et al, 2007). GABA$_B$ receptors were down-regulated in the dorsal horn, which could also contribute to hyperactivity in these neurons and neuropathic pain (Wang et al, 2011).

The effect of exercise on GABA receptors in the DRG are not known.

Adrenoreceptors

The α1A-adrenergic receptors are functionally expressed in capsaicin-sensitive, nociceptive sensory neurons. Activated receptors inhibited neurogenic inflammation (Trevisani

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et al, 2007). It has also recently been shown that when these receptors were stimulated for long periods of time, there was enhanced neurogenesis (Gupta et al, 2009) and synaptic plasticity in the hippocampus leading to improved cognitive function, mood and longevity (Doze et al, 2011). It is not known how exercise effects these receptors in the DRG.

Glutamate Receptors

Glutamate receptors are present in both pre-synaptic primary afferent terminals, and post-synaptic dorsal horn neurons where they mediate excitation (Sato et al., 1993; Liu et al., 1994; Ma & Hargreaves, 2000; Marvizon et al., 2002; Li et al., 2004; Nagy et al., 2004; McRoberts et al., 2007). Glutamate receptors acting at primary afferent terminals were involved in pain transmission by increasing SubP (Marvizon et al., 1997; Malcangio et al., 1998; Marvizon et al., 1999; Chen et al., 2010; Liu et al., 1997) and glutamate release (Yan et al., 2013). During neuropathic pain, NMDA receptors were potentiated, which may contribute to hyperalgesia (Chen et al, 2014). We wanted to examine if Glur2, 3 and/or NR1 pre-synaptic glutamate receptors were modulated by exercise, and therefore could contribute to exercise analgesia.

Serotonin (5HT) Receptors

5HT receptors in the spinal cord, both pre- and post-synaptic to primary afferent terminals, are activated by the descending 5HT system, which contributes to anti-nociception (Yoshimura & Furue, 2006; Laporte et al, 1995). Additionally, 5HT receptors are located on peripheral nerve terminals where they act to modulate the pain response (Taiwo & Levine, 1992; Andrews & O'Neill, 2011). 5HT release can be activated via cortical stimulation (Viisanen &

Pertovaara, 2010), exercise (Hoffmann et al, 1990) and injured nerve and inflammatory states (Liu et al, 2002).

Figure 4: Serotonin (5-HT) receptor families and subtypes
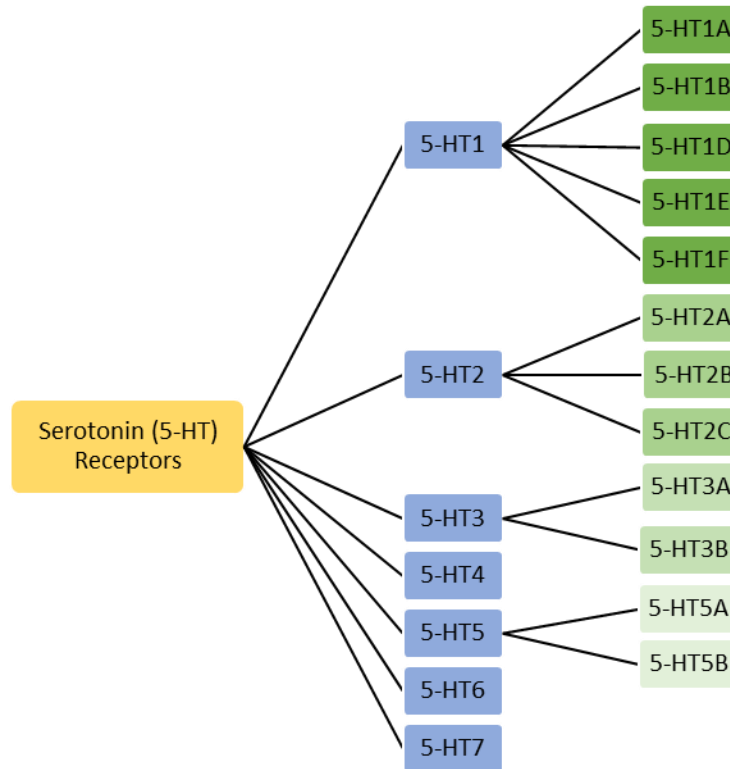
5HT1A receptor activation in C-type nociceptive neurons inhibited high threshold calcium currents leading to decreased action potential duration and steady state inhibition (Cardenas et al, 1997). Todorovic & Anderson (1992) found that 5HT acting on the 5HT1A receptor caused hyperpolarization in Aδ and C-fibers. Furthermore, peripherally acting 5HT1A receptors resulted in a reduction of chronic pain (Andrews & O'Neill, 2011).

5HT1D receptors were found on peptidergic nociceptors (Potrebic et al, 2003) in addition to medium and large DRG neurons (Nicholson et al, 2003), and have been implicated as a target

in migraine treatment (Potrebic et al, 2003). Furthermore, 5HT1D/1B receptor agonists were involved in both headache and non-headache pain (Dussor, 2014). 5H1F and 5HT7 receptor agonists were more recently discovered to be potential migraine treatments (Agosti, 2007). 5HT7 receptor activation played an inhibitory role in spinal pain modulation, whereas 5HT3 receptors acted to facilitate pain (Dogrul et al, 2009). In contrast, an earlier study showed the 5HT3 receptor inhibited nociceptive transmission in dorsal horn neurons post-synaptically via evoking GABA release from primary terminals (Alhaider et al, 1991).

5HT2A receptors were mainly expressed in IB4 and TRPV1 containing nociceptive sensory neurons, and were concentrated in the cytoplasm and on the plasma membrane, suggesting a functional role at these sites (Van Steenwinckel et al, 2009). There was also evidence to support the 5HT2A receptors' role in peripheral control of sensory afferents and cellular excitability of some cell bodies (Van Steenwinckel et al, 2009). This receptor was also involved in inflammatory pain and hyperalgesia (Wei et al, 2005) to radiant heat (Tokunaga et al, 1998) and mechanical stimuli (Chen et al, 2006). Furthermore, both 5HT2A and 5HT2B receptors were implicated in spinal hyper-excitation which contributes to neuropathic pain (Aira et al, 2010; Van Steenwinckel et al, 2008) and mechanical hyperalgesia (Lin et al, 2011).

The role of the 5HT2C receptor is less clear. One author suggested that the 5HT2C receptor may be specifically induced in lumbar DRG by inflammatory pain (Nicholson et al, 2003). However, the 5HT2C receptor modulated neuropathic pain (Panczyk et al, 2015; Baptista-de-Souza et al, 2014) as well as mechanical hyperalgesia associated with diabetic neuropathy (Bektas et al, 2014) at the spinal level.

There is discrepancy as to the serotonin (5HT) receptor subtypes detected in the DRG. All 15 subtypes have been investigated using various methods. 5HT receptors 1B, 1D, 2A, 3 and

4 have been confirmed in the lumbar DRG of rats (Pierce et al, 1996; Wu et al, 2001; Nicholson et al, 2003; Classey et al, 2010). However, there is conflicting evidence for other 5HT receptor subtypes present in rat lumbar whole DRGs. For instance, Pierce et al (1996) performed PCR to detect mRNA for receptors 2C and 7, but not for 1A, 1F, 2B or 5A. In another study utilizing RT-PCR, receptors 1A, 1F, 5A and 7 were present, but 2B and 2C were not. More recently in 2010, Classey et al. showed dense staining of the 1F receptor using immunohistochemistry. Nicholson et al (2003) performed in situ hybridization to identify 2B positive neurons present in all small, medium and large cells, but did not display any detection of 1A, 1E, 2C, 5A or 7 receptors. Furthermore, receptors 1B and 4 were more highly expressed in medium neurons, 1D and 3B in large neurons, and 2A in small neurons. These discrepancies amongst studies reveal the variability associated with gene expression results for 5HT receptors in lumbar whole DRGs in the rat. Therefore, four of these receptors are controversial in terms of detection in the DRG (i.e. 5HT1A, 1F, 2C, 7). Hence, our study will aid in resolving this conflict by using the novel technique of LCM to isolate single small and large DRG cells, and then use the highly sensitive qPCR method to reliably quantify 5HT receptor mRNA.

In summary, 5HT can activate various 5HT receptors that are produced and act in DRG neurons to regulate the pain response. Change in number, or responsiveness, of these receptors can alter pain transmission. How exercise and diabetes effects 5HT receptors in the DRG is not known.

Tropomyosin Related Kinase (Trk) Receptor

In adult DRG neurons, TrkA, B and C are found in varying amounts depending on the neuron size, peripheral target tissue the neuron projects to, co-expression of neurochemical markers as well as the type of signaling the neuron is involved in.

Humans expressed TrkA in 46%, TrkB in 29% and TrkC in 24% of DRG neurons (Josephson et al, 2001). Rats expressed TrkA in 35-45%, TrkB in 5-26% and TrkC in 15-28% of DRG neurons (Kashiba et al, 1996; Wetmore & Olson, 1995; Kobayashi et al, 2005). However, depending on the sensory target, DRG neuron composition varies (Lu et al, 2001; Castaneda-Corral et al, 2011). For example, neurons that project to the lateral gastrocnemius muscle were 20% TrkA, 50% TrkB and 73% TrkC positive, whereas neurons associated with the visceral pelvic nerve were 90% TrkA, 94% TrkB and 2% TrkC positive (McMahon et al, 1994). This example also shows that there is overlap in Trk receptor localization amongst subsets of DRG neurons. For instance, TrkB is co-localized with TrkC in neurons projecting to muscle (McMahon et al, 1994). Neurons innervating skin have a majority (80%) of TrkA positive cells, neurons innervating muscle contain a majority of TrkC positive cells, and a small percentage of cutaneous and visceral pelvic innervating neurons contain a majority of TrkB positive cells that may also co-express TrkA (Ernsberger, 2009).

Small DRG neurons preferentially expressed TrkA receptors (Mu et al, 1993; McMahon et al, 1994). Furthermore, 92% of these TrkA positive neurons also expressed CGRP in the rat (Averill et al, 1995). Hence, the bulk of this subpopulation of DRG neurons are nociceptive in nature, although not all small TrkA positive cells are involved in pain signalling. TrkA activation enhanced acute and chronic pain, whereas inhibition of TrkA signalling decreased pain (Mantyh et al, 2011; Chen et al, 2014). Furthermore, anti-TrkA antibody treatment in mice resulted in decreased inflammatory and neuropathic pain (Ugolini et al, 2007). Although TrkA receptors were mainly expressed in peptidergic C-fiber neurons (Snider & McMahon, 1998), they were found in large A fibers as well (Fang et al, 2005).

Large DRG neurons preferentially expressed TrkC (Mu et al, 1993; McMahon et al, 1994), and proprioceptors relied on TrkC signalling (Ernsberger, 2009). TrkC mRNA was increased in DRG neurons after nerve injury and modulated neuropathic pain (Tender et al, 2011). It is not clear if medium-sized neurons preferentially express TrkB receptors as they are present on each size classification of neuron; however, certain DRG neurons do depend solely on TrkB signalling as well as its co-expression with other Trk receptors (Ernsberger, 2009; Wright & Snider, 1995; Acheson et al, 1995). After CFA injection inducing mechanical hyperalgesia, there is an increase in TrkB mRNA and protein in DRG cells (Lin et al, 2011). Alternatively, not all DRG neurons express TrK receptors (McMahon et al, 1994).

Diabetes resulted in decreased TrkA mRNA levels in lumbar whole DRG (Delcroix et al, 1998; Maeda et al 1996) but there was no change in protein levels or axonal transport of TrkA (Delcroix et al, 1998; Delcroix et al, 1997).

To our knowledge, no studies have been completed examining the effect of exercise on Trk receptor expression in healthy or diabetic small and large DRG neurons.

Growth-related Genes

Neurotrophins were examined in both study 1 and 2. Neurotrophins are unique because they are retrogradely transported from their target cells, which influence their sensory neurons where the corresponding receptors are located (Levi-Montalcini, 1987; Barde, 1989). Neurotrophins and their signaling pathways are also involved in sensory neuropathy (Anand, 2004; Walwyn et al, 2006).

BDNF and NT3 were both shown to increase in expression in whole DRG (Molteni et al, 2004) and spinal cord (Gomez-Pinilla et al, 2001) after 7 days of voluntary wheel running. BDNF also increased in DRG in response to spinal nerve ligation (Fukuoka et al, 2001). NT3 was decreased in muscle of animals with diabetic neuropathy (Fernyhough et al, 1998), but its increase was involved in the decrease of muscle derived pain (Gandhi et al, 2004).

NGF was shown to regulate nociceptive responses to heat (Shu & Mendell, 1999). NGF also plays a role in diabetes. It was involved in diabetic nerve regeneration (Xu & Sima, 2001), and was associated with thermal hypoalgesia and conduction slowing of large sensory fibers in diabetes (Diemel et al., 1994; Fernyhough et al., 1995; Apfel et al., 1994). NGF was used to treat diabetes-induced hypoalgesia (Christianson et al, 2003) and was also necessary for structural protein integrity in the DRG soma (Hellweg & Hartung, 1990).

IGF1 was preferentially expressed in small DRG neurons, and it was decreased in these neurons of STZ rats (Craner et al, 2002). When systemic IGF1 was artificially increased in animals with DPN, it reversed hypoalgesia and lead to improved locomotion (Chu et al, 2008), caused sensory nerve regeneration (Ishii & Lupien, 1995), restored thermal sensitivity, and returned peripheral nerve conduction velocity to normal (Piriz et al, 2009).

Gap-43 is associated with enhanced axonal outgrowth (Mohiuddin et al, 1995). GAP-43 was up-regulated in whole DRGs after short-term voluntary exercise, and there was also an increase in neurite outgrowth (Molteni et al, 2004). GAP-43 mRNA levels were decreased in whole DRGs of STZ rats (Mohiuddin, 1995). GAP-43 levels were also decreased in iENF C-fibers in the skin of patients with early Type 2 diabetes, indicating impaired regeneration of these fibers and a marker for DPN (Bursova et al, 2012). However, an increase in GAP-43, associated with enhanced regeneration of iENFs, in the skin of patients with painful DPN was distinguished

from patients with non-painful DPN, who did not show an increase in GAP-43 (Cheng et al, 2013).

How exercise effects the expression of BDNF, NT3, NGF, IGF-1 and GAP43 in small and large DRG neurons is not known.

Synaptic Vesicle-related Genes

VGlut's are essential for vesicular uptake of glutamate from the extra-cellular fluid (Danbolt, 2001). These transporters are vesicle-bound and, more specifically, mainly associated with synaptic vesicle membranes (Shigeri et al, 2004). VGlut2 was found in a high percentage of small to medium sized DRG neurons that co-expressed CGRP and IB4 (Brumovsky et al, 2007). Hence, all DRG neuron sub-types of adult mice use glutamate as a transmitter (Brumovsky et al, 2007). However, neuropeptides act as neurotransmitters and are released from afferent terminals as well, such as CGRP and SubP (please refer to neuropeptides section).

Synapsin I is present in synaptic vesicle membranes of the central and peripheral nervous system and acts to enhance neurotransmitter release. This gene was also upregulated after voluntary exercise in rats (Molteni et al, 2004).

Syntaxins (STX) help dock synaptic vesicles to the presynaptic plasma membrane in order to permit neurotransmitter release. STX1A, a member of the STX family, is found in dorsal root nerve fibers as well as in the plasma membrane of DRG neurons (Aguado et al, 1999). It inhibited N-type calcium channels in chick DRG neurons (Lu et al, 2001), was involved in synaptic plasticity in the nociceptive pathway (Takasusuki et al, 2007), and exhibited anti-nociception via CGRP release from sensory neurons (Meng et al, 2007).

The effect of exercise on the expression of VGlut2, SYN1 and STX1A and 1B mRNAs in small and large DRG neurons is not known.

Neuropeptides

CGRP is a member of the calcitonin family, is a potent peptide vasodilator (Brain et al, 1985) and functions in pain transmission (Kuraishi et al, 1988). SubP is a member of the tachykinin neuropeptide family, and also plays a role in pain transmission (De Felipe et al, 1998; Zubrzycka & Janecka, 2000). Both neuropeptides are released and act both centrally and peripherally. CGRP has been found in all DRG neuron cell sizes; however, it was preferentially expressed in small neurons (Averill et al, 1995). CGRP seems to be NGF dependent, as a nerve injury decreased the amount of NGF reaching the DRG, leading to a decreased amount of CGRP produced in DRG neurons (Doughty et al, 1991). Both CGRP and SubP positive neurons atrophied and were decreased in type 1 diabetes (Bennett et al, 1998). Both neuropeptides were also decreased in small nerve fibers of rats with DPN who experienced a decrease in sensory nerve function (Chen et al, 2005).

The endogenous opioid system is involved in pain processing, analgesia, as well as the perception of non-painful somatosensory stimuli (Mueller et al, 2010). The δ-opioid receptor (OPRD1) was expressed in large and small DRG cells, coexisting with the μ-opioid receptor (OPRM1) in peptidergic small DRG neurons (Wang et al, 2010). OPRD1 and OPRM1 agonists' caused an attenuation of depolarization induced calcium currents in small DRG neurons, hence, inhibited C-fiber synaptic transmission in the dorsal spinal cord (Wang et al, 2010). STZ mice had an attenuated analgesic response to an OPRM1 agonist (Kamei et al, 1992), whereas in STZ

rats, both receptors were involved in the hypoalgesic response (Kolta et al, 1996). Opioids also play a role in the increased pain threshold associated with exercise (Koltyn, 2014; Galdino et al, 2010) and the positive effects of exercise on diabetes (Stagg et al, 2011). Interestingly, in non-human primates, 5HT uptake inhibitors increased the anti-nociceptive effects of an OPRM1 agonist (Banks et al, 2010).

The effect of exercise on the expression of CGRP, SubP, OPRD1 and OPRM1 mRNAs in small and large DRG neurons is not known.

Summary of Gene Categories

In summary, based on the preceding background information on the genes included and their relevance in this thesis, the main gene categories focused on in each study are presented in Table 1. These particular genes were chosen because of their relevance to nociceptive and proprioceptive sensory processing. Each of these genes has potential functional relevance from exercise, pain, locomotor and DPN perspectives.

For the first study, we did not know what to expect in terms of small and large DRG neuron gene expression changes with acute treadmill exercise. Therefore, we chose a broad range of genes to investigate what we considered as important groups of genes: ion channels, GABA and adrenergic receptors, glutamatergic receptors, serotonergic receptors, tropomyosin related kinase receptors, growth related and synaptic vesicle related. Since these groups of genes are all evident in the DRG, and some of them were previously shown to be differentially expressed in small and large DRG neurons of healthy, non-exercised animals, we wanted to determine if exercise and diabetes affected their expression.

Based on the results from the acute exercise exploratory study, and in preparation to compare to the diabetic chronic exercise study, the second part of study 1 (i.e. chronic exercise in healthy rats) included additional genes. Since the 5HT1A receptor and Nav1.2 were the only two mRNAs that indicated a difference in levels due to acute exercise, other 5HT receptors and ion channels were added in subsequent studies. Because of the role of the 5HT1A receptor in anti-nociception, additional pain-related genes were included. IGF-1 and GDNF were also added due to their response to exercise and relevance in DPN.

Table 1: Genes tested using qPCR

	Study 1 - acute	Study 1 - chronic	Study 2
Ion Channels	$Na_v1.2$/Scn2a	$Na_v1.2$/Scn2a	$Na_v1.8$/Scn10a
	$Na_v1.6$/Scn8a	$Na_v1.3$/Scn3a	TRPV1
	$Na_v1.9$/Scn11a	$Na_v1.6$/Scn8a	
	$K_v1.1$	$Na_v1.7$/Scn9a	
	$K_v1.2$	$Na_v1.8$/Scn10a	
	$K_{Ca}2.2$	$Na_v1.9$/Scn11a	
	$K_{Ca}2.3$	TRPV1	
GABA & Adrenergic Receptors	$GABA_{A\alpha2}$		
	$GABA_{A\beta3}$		
	$GABA_{A\gamma2}$		
	$GABA_{B1}$		
	$GABA_{B2}$		
	$ADRA_{\alpha1A}$		
	$ADRA_{\alpha1D}$		
Glutamatergic Receptors	GluR2		
	GluR3		
	NR1		
Serotonergic Receptors	$5HT_{1A}$	$5HT_{1A}$	$5HT_{1A}$
	$5HT_{2A}$	$5HT_{1B}$	$5HT_{1F}$
	$5HT_{2C}$	$5HT_{1D}$	
	$5HT_7$	$5HT_{1F}$	
		$5HT_{3A}$	
Tropomyosin Related Kinase Receptor	TRKA	TRKA	TRKA
	TRKB	TRKB	TRKB
	TRKC	TRKC	TRKC
Growth Related	BDNF	BDNF	BDNF
	NGF	NGF	NGF
	NT3	NT3	NT3
	Gap-43	Gap-43	Gap-43
		IGF1	IGF1
			GDNF
Synaptic Vesicle Related	SYN1	SYN1	
	STX1A	STX1A	
	STX1B		
	VGLUT2		
Pain Related		CGRP	CGRP
		SubP	SubP
		OPRD1	
		OPRM1	
		CNR1	

GENERAL METHODS

Our experimental approach was similar for both of the studies that make up this thesis. I will explain in detail the basic approach, and then further clarify differences based on the particular study.

Female Sprague-Dawley rats were obtained from the University of Manitoba colony according to their weight (275-300g – approx. 13 weeks old). Female rats were chosen due to their lower weight gains with age (http://www.taconic.com/rat-model/sprague-dawley) and thus, one would expect less differences in body weight between sedentary and exercised animals. Our study required a sample size of at least 2 animals, but based on the novelty of our particular experiments, we used a minimum n-value of 4 animals per study (Hassard, 1991; see appendix 7 for details). Treadmill animals were housed in pairs (1 control and 1 exercised rat) in standard rat cages with access to food (5P00 Prolab RMH 300 diet consists of crude protein not less than 22.0%, crude fat not less than 5.0%, crude fiber not more than 5.0 %, Ash not more than 6.0%, and added minerals not more than 2.5%) and water Ad libitum. There was a 12 hour light-dark cycle in the animal room. The animals began exercise one week following their arrival. Animals were sacrificed via decapitation 4 hours after the last exercise bout. L4 and L5 DRGs from both sides were quickly removed and flash-frozen in optimal cutting temperature (OCT) compound (Tissue-Tek) at -80°C. DRGs were then warmed to -20°C in the cryostat (Shandon), and the OCT block was mounted onto the chuck and sectioned at 12um onto Fisherbrand Superfrost precleaned microscope slides (Catalog # 12-550-016; size 25 x 75 x 1.0mm). After 6 sections were lifted per slide, they were placed in a slide box with desiccant and stored at -80°C. It took approximately 2-5 minutes to section 1 slide depending on the tissue.

Ethics approval for all studies was obtained from the University of Manitoba Animal Ethics Committee.

Laser Capture Microdissection (LCM)

The DRG sections were stained and fixed, through a series of hydration and dehydration steps, to avoid RNA degradation (LCM staining kit from Ambion; AM1935). The slides were transferred from the -80°C freezer to the cryostat, set at -20°C, to warm them prior to staining (for 15 minutes). Next, the slides were placed directly into 100% ethanol (cooled to -20°C in a freezer) for 1 minute. The slides were then successively dipped into 95% ethanol, 75% ethanol, and 50% ethanol for 6-7 dips each. The tissue section was then stained with cresyl violet (4% cresyl violet in 75% ethanol) for 20-60 seconds. Subsequently, the slides were placed in containers with 50% ethanol, 75% ethanol and 95% ethanol successively for 5 seconds each. Then, the slide was placed into 100% ethanol (which had been subjected to dehydration beads) for 30 seconds, followed by a new 100% ethanol solution for 1 minute. After a quick rinse in xylene (5-7 dips), the slide was left in another xylene solution for 5-15 minutes to dehydrate. The slides were air dried for 5 minutes and then put into a vacuum desiccator for 30 minutes.

LCM was then immediately performed on the sections (small <= 30um; large >= 40um; Applied Biosystems Arcturus LCM system; Please see Figure 5). We measured the neurons with an on-screen, digital ruler, and made sure each cell body captured had a visible nucleus (Figure 5: B). A timer was set to 20 minutes for cell collection (minimum of 500 per size category; Figure 5: D-E) via the infrared laser and then the cap (Figure 5: F) was placed onto a tube with 20ul of lysis buffer and placed in a heater (set at 42°C for 30 minutes) with cap facing downward. Then the cap was discarded and the tube closed (filled with lysis buffer and the cells

that were removed from the cap) and placed in a -80°C freezer. If multiple tubes were collected

from the same animal, all tubes were combined prior to RNA isolation.

Figure 5: Illustration of LCM of DRG cells.
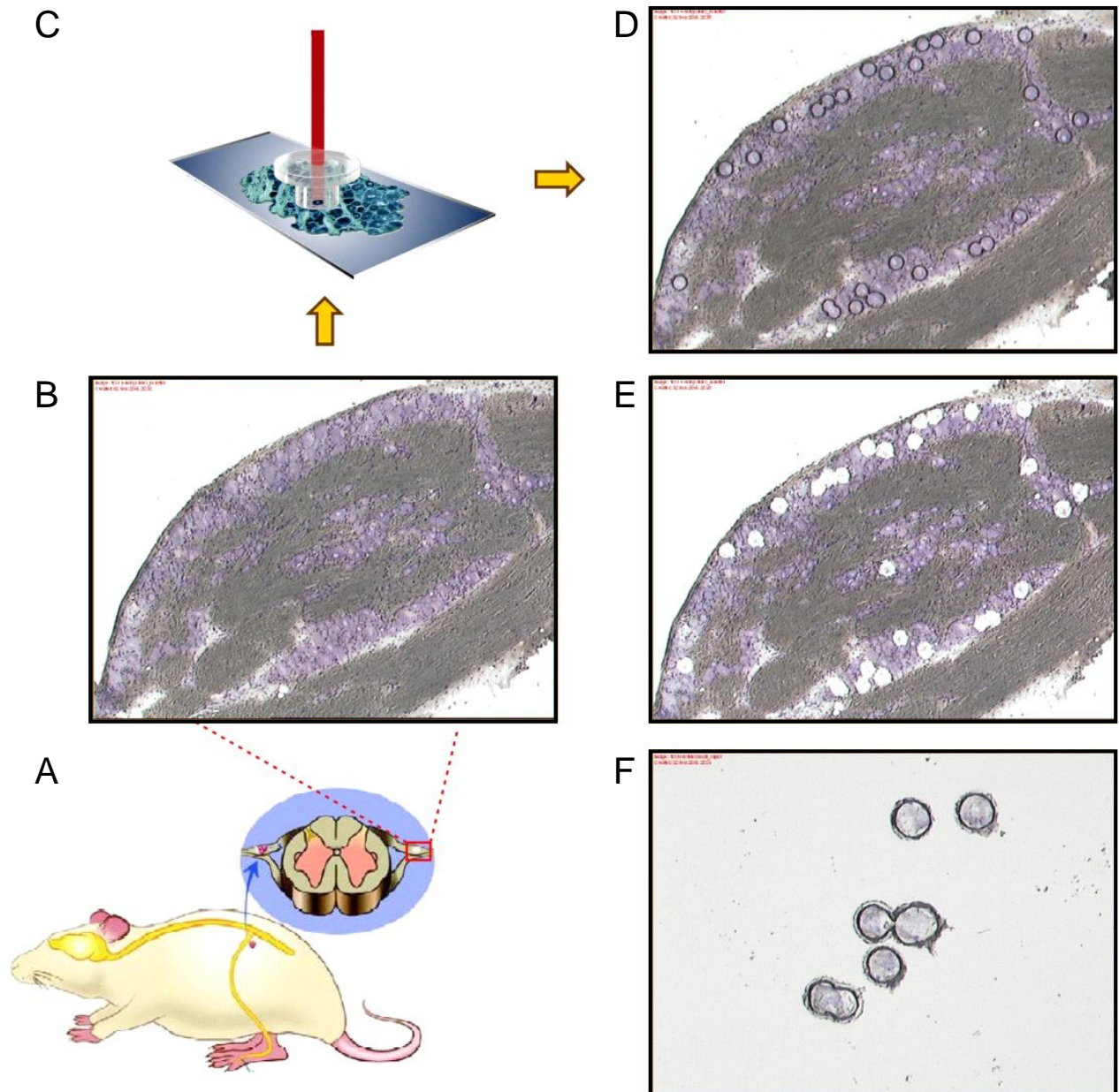
Legend: A) rat DRG B) microscope view of single DRG section on slide at 20x magnification C) LCM cap placed on DRG section on slide. Showing infrared laser adhering the desired cell to the cap D) DRG section with large cells selected with laser E) section after cap was lifted and large cells gone F) cap with large cells on it.

RNA Isolation & Testing

Total RNA was then isolated from combined samples of large (>=900 cells) and small (>=700 cells) DRG neurons (Ambion RNAquesous Micro Kit) and tested for RNA integrity and concentration values (Agilent 2100 Bioanalyzer). We chose this particular isolation kit because it is specific to low LCM samples for optimal RNA recovery. The kit is based on the spin column-based nucleic acid purification extraction method. This method involves the sample being lysed (guanidinium thicyanate solution), mixed with ethanol, and then added to a column equipped with a silica-based filter that selectively binds RNA. The column is then washed and eluted to give purified RNA. More specifically, the columns in this kit have a very small filter to enable RNA to be eluted in small volumes (10-20ul), and hence, lead to more concentrated RNA and greater RT-PCR sensitivity. In addition, this kit contains a post-elution DNase treatment to ensure no genomic DNA is present for RT-PCR assays.

The bioanalyzer was then used with the Agilent RNA 6000 Pico Kit to determine RNA integrity (Schroeder et al, 2006). This method is based on microcapillary electrophoretic (electrolyte filled capillary helps separate species based on their size to charge ratio) RNA separation and measurement, followed by an extracted algorithm that calculates the RNA integrity number (RIN; reflection of degree of degradation). The RIN algorithm is more consistent and accurate at conveying integrity values to RNA measurements than other traditional methods. RNA integrity is important for these experiments since it reflects gene expression at the instant of RNA extraction. A RIN of 6.5 or better was used as criterion for proceeding with cDNA synthesis.

cDNA Synthesis & Pre-amplification

  Samples were then reverse transcribed to cDNA (SuperScript VILO cDNA synthesis kit by invitrogen) and preamplified (TaqMan PreAmp Master Mix from Applied Biosystems) for qPCR preparation. The protocol used for first-strand cDNA synthesis can be found in Appendix 1. Two enzymes, reverse transcriptase (superscript III in this case) and DNA polymerase, were used as catalysts for the reaction of DNA made from a mRNA template. This particular kit provides better cDNA synthesis efficiency and can be used with very low RNA input amounts, leading to a linear response in message abundance (ie. measured by RT-qPCR).

  The pre-amplification reactions were mixed together and placed in a thermocycler according to protocol in Appendix 1. Since RNA amounts are relatively low for single cell-pooled analysis, performing a pre-amplification step improves sensitivity of RT-qPCR. This is evident particularly for low abundance genes, resulting in substantially higher cDNA amounts. Furthermore, incorporating a pre-amplification step also allowed us to amplify only target genes, which we could then measure quantitatively with RT-qPCR. Because only a limited dilution of primers was used, and just 14 cycles of PCR were performed, the reaction does not enter the plateau phase. Essentially, by pre-amplifying we greatly increase the transcripts, without affecting the relationships between them, improving the Ct. The Ct is the "number of cycles required for the fluorescence signal to cross the threshold" and is a relative measure of the concentration of the target gene in the PCR reaction (i.e. a lower Ct equals a higher amount of target nucleic acid in the sample) (www.researchgate.net; www.appliedbiosystems.com).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR is a precise, reproducible and highly sensitive tool to determine expression of our genes of interest in small and large DRG cells. The ability to quantitatively measure RNA results in a direct measure of cellular activity via gene expression. Pre-amplified cDNA samples were prepared for real-time polymerase chain reaction experiments (Applied Biosystems 7500 Real-Time PCR system) according to Appendix 1. Compared to traditional PCR, qPCR not only amplifies the targeted DNA molecule, but additionally detects the product in real time as the reaction progresses versus at the end. We used sequence-specific DNA probes (increasing specificity) that emit fluorescence that is detected only after it has been annealed to its complementary sequence and then cleaved by the DNA polymerase (Taq), to quantify mRNA. In particular, the PCR products were quantitatively synthesized from cDNA samples using the TaqMan Gene Expression Master Mix for each gene we studied. Therefore, each subsequent PCR cycle results in an increase in the product, causing an increase in fluorescence. This increase is used to determine the threshold cycle (C_t) in each reaction.

Figure 6: 2-step qPCR

Extension of primer on mRNA
RT Step
Synthesis of 1st cDNA strand

PCR Step
Extension of primer on cDNA
Completion of 2nd cDNA strand
PCR amplification of cDNA
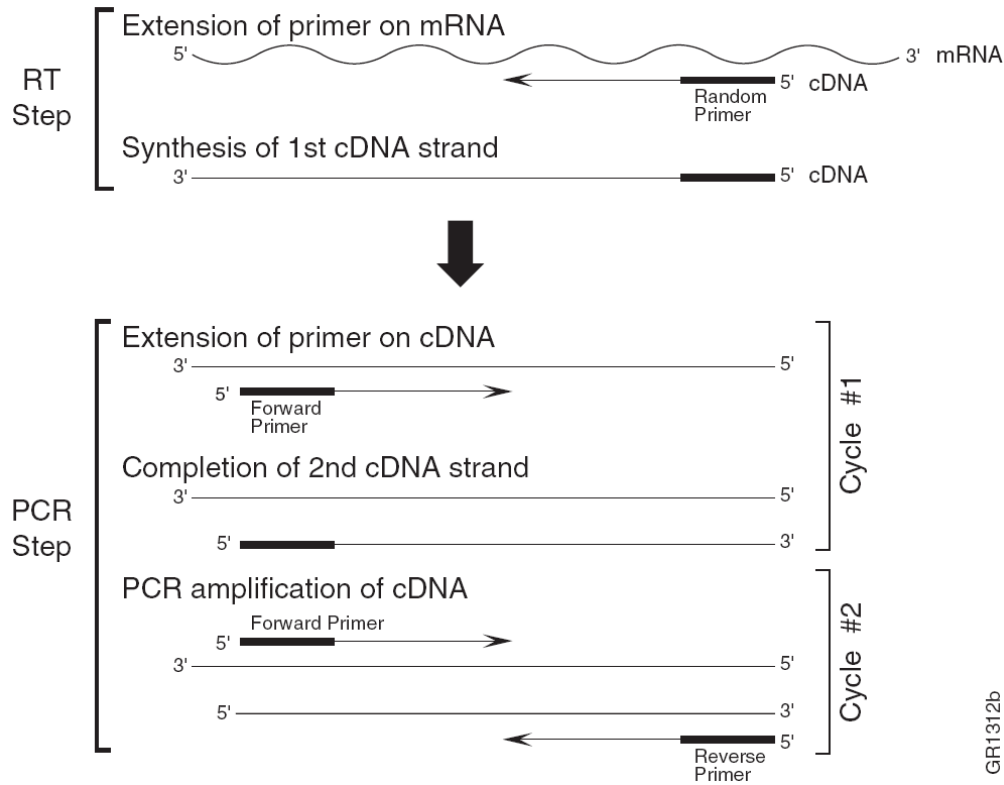
(Applied Biosystems Protocol, 2010)

Our protocol consisted of a series of temperature changes (cycles) that involved three main steps: 1) 1 cycle of 50°C for 2 minutes (UDG incubation/optimal UDG enzyme activity) 2) 1 cycle of 95°C for 10 minutes (activate Taq polymerase) 3) 40 cycles of 95°C for 15 seconds (dissociate template strand from the sample cDNA) followed by 60°C for 1 minute (annealing of probe and primers to target cDNA / polymerase extends primers and cleaves probe).

Table 2: The real-time qPCR thermal cycling conditions.

Step	UDG Incubation	Enzyme activation	PCR	
			Cycle (40 cycles)	
	HOLD	HOLD	Denature	Anneal/Extend
Time	2 min	10 min	15 sec	1 min
Temperature	50°C	95°C	95°C	60°C

Each target gene was compared to the internal control gene (ie. housekeeping gene; Succinate dehydrogenase complex subunit A (SDHA)) for relative quantification of mRNA. This particular internal control was chosen due to previous reference gene studies in exercised animals that suggested SDHA was the most reliable out of the genes tested (Cappelli et al, 2008), as well as our own tests to verify SDHA would work as an internal control gene on rat nervous tissue and was the most reliable (Woodrow et al, 2013). The external control for Study 1 & 2 was whole lumbar spinal cord. For study 3 we used whole lumbar DRG.

Slide staining, LCM, RNA isolation, cDNA synthesis, pre-amplification and qPCR were performed by Natasha Paddock. DRG sectioning and RNA integrity analysis were performed by Patricia Sheppherd.

Table 3: Example of PCR reaction plate set-up (triplicate format)

Small DRG	1	2	3	4	5	6	7	8	9	10	11	12
TE37	SDHA	SDHA	SDHA	Scn2a	Scn2a	Scn2a	Htr1a	Htr1a	Htr1a	Synl	Synl	Synl
TE38	SDHA	SDHA	SDHA	Scn2a	Scn2a	Scn2a	Htr1a	Htr1a	Htr1a	Synl	Synl	Synl
TE39	SDHA	SDHA	SDHA	Scn2a	Scn2a	Scn2a	Htr1a	Htr1a	Htr1a	Synl	Synl	Synl
TE40	SDHA	SDHA	SDHA	Scn2a	Scn2a	Scn2a	Htr1a	Htr1a	Htr1a	Synl	Synl	Synl
TE41	SDHA	SDHA	SDHA	Scn2a	Scn2a	Scn2a	Htr1a	Htr1a	Htr1a	Synl	Synl	Synl
TE42	SDHA	SDHA	SDHA	Scn2a	Scn2a	Scn2a	Htr1a	Htr1a	Htr1a	Synl	Synl	Synl
Water	SDHA	SDHA	SDHA	Scn2a	Scn2a	Scn2a	Htr1a	Htr1a	Htr1a	Synl	Synl	Synl
Lumbar SC	SDHA	SDHA	SDHA	Scn2a	Scn2a	Scn2a	Htr1a	Htr1a	Htr1a	Synl	Synl	Synl

Exercise Protocols

There were two exercise protocols utilized: the voluntary wheel and the treadmill. The voluntary wheel was the large rodent activity wheel and living space (Model 80859L; Lafayette Instrument Company). The wheel was connected to a monitoring computer that recorded wheel data for each animal.

The treadmills were obtained from Columbus Instruments (model Exer 3/6) with 3 individual lanes/belts for rats and electrical stimulus with individual on/off switches per lane. Treadmills were covered with a towel to make it dark for the rats so they were more comfortable. Also, shocks were only used initially in the training program and was complemented with a pair-timed tap to the side of the running lane so that subsequently only a slight tap was required to keep them running. An edible treat was provided to each animal post-run. Animals were run the same time of day, each day, during the rats wake cycle.

Study 1 treadmill exercise was supervised by Lindsey Woodrow and Jeremy Chopek. Study 1 voluntary wheel exercise supervision was carried out by Kalan Gardiner and Natasha Paddock. Study 2 voluntary wheel exercise was supervised by Marc Morissette.

Figure 7: Exercise machines. A. Voluntary Wheel. B. Treadmill.
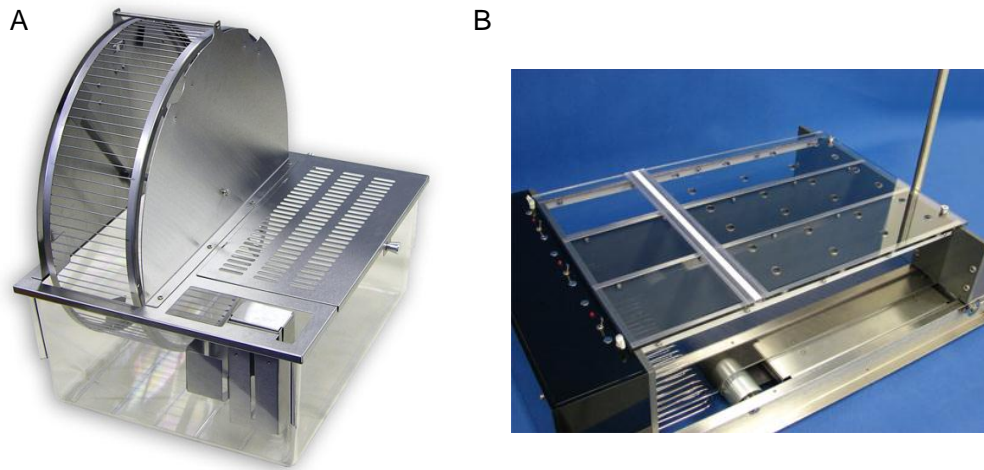
Statistical Analysis

The results of qPCR experiments were expressed in relative quantification (RQ) values as calculated by 7500 Software version 2.0 (Applied Biosystems) using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). We calculated the average RQ for each gene analysed, then we compared the sedentary vs. treatment values for each gene (i.e. % RQ). mRNA levels under treatment conditions (i.e. exercise and STZ) are expressed as a percentage relative to the healthy sedentary animals (i.e. healthy sedentary represented at 100%). Two-tailed independent Student's t-tests were used to compare RQ values between exercised and control groups for each target gene in the acute study of Study 1. For the remaining studies where 3 groups were compared, one-way ANOVA was used followed by the Tukey post-hoc test to show all pairwise multiple comparisons. A statistically significant difference for all studies was defined as $P < 0.05$.

Study 1: The Effect of Acute and Chronic Increases in Neuromuscular Activity on

Gene Expression in Small and Large Dorsal Root Ganglion Neurons: Healthy Rat

ABSTRACT

Dorsal root ganglion (DRG) neurons are responsive to altered neuromuscular activity. Previous research has shown that the expression of several genes are up-regulated in healthy whole DRG after acute voluntary wheel exercise. Understanding the effect of exercise on gene expression in small and large DRG neurons, in healthy rats, may provide insight into mechanisms involved in changes to sensory processing associated with increases in neuromuscular activity. We present evidence that small and large DRG neurons are differentially affected by exercise.

We examined gene expression in samples of small and large cells of the L4/L5 lumbar DRG and their specific response after exercise to identify potential molecular processes involved in activity-dependent changes. DRGs were collected 4 hours after the last exercise session, and RNA was isolated from samples of large and small DRG cells following laser capture microdissection. Relative mRNA levels were determined using real-time polymerase chain reaction experiments.

Healthy adult rats received treadmill exercise for 1 or 17 weeks, or voluntary wheel exercise for 16 weeks. Control animals were healthy and sedentary. Gene expression analysis focused on ion channels, GABA and adrenergic receptors, glutamatergic receptors, serotonergic receptors, tyrosine kinase receptors, growth-related, synaptic vesicle-related and pain-related genes because of their potential roles in nociception and proprioception.

Sedentary rats displayed higher expression in small relative to large cells for 19 genes, higher expression in large compared to small cells for 15 genes, and no difference in expression between small and large cells for 12 genes, out of the 46 studied.

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In small and large DRG cell samples, one week of treadmill exercise did not significantly alter the expression of mRNA for any genes examined. Following chronic treadmill or voluntary wheel exercise, gene expression responses in small and large DRG cells varied considerably between exercise types. In small DRG neuron samples, chronic treadmill exercise increased the expression of mRNA for 5HT1D and decreased expression for 5HT1F receptors. In large DRG neuron samples, chronic treadmill exercise decreased the expression of mRNA for 5HT1A and TrkC receptors, and Syn1. In small DRG neuron samples, chronic voluntary wheel exercise decreased the expression of mRNA for 5HT1D, TrkA and OPRD1 receptors and increased expression for GAP43. In large DRG neuron samples, chronic voluntary wheel exercise increased the expression of mRNA for 5HT3A and GAP43 and decreased expression for 5HT1D, Nav1.6, OPRD1, TrkA, TrkC and Syn1. Small and large DRG neurons respond differently to the duration and intensity of exercise. No clear pattern relating to the responsiveness of particular groups of expressed genes was apparent in any condition. DRG neurons seem to respond to chronic increases in neuromuscular activity. DRG neurons show a greater response to voluntary exercise, with less distance travelled and less intensity during the training period, compared to forced exercise.

Our results demonstrate that exercise affects expression of genes involved in activity-dependent neural plasticity differentially in small and large DRG neurons, and that these change with prolonged and less intense periods of exercise. For the genes tested, large neurons were more affected than small neurons in healthy animals after chronic exercise. The results suggest potential new mechanisms for analgesia associated with exercise, among other nociceptive and proprioceptive implications.

RATIONALE AND PURPOSE

This study comprises an investigation into the differences in gene expression of small and large DRG neurons, in healthy rats, in response to exercise. It was previously demonstrated that the expression of BDNF, NT3, Syn1 and GAP-43 were up-regulated in healthy, non-injured rat DRGs after 7 days of voluntary exercise (Molteni et al., 2004). We wished to extend these observations to include forced treadmill exercise, add expression of other physiologically relevant genes, add a longer period of exercise, and determine the mRNA levels of each gene in small ($<=30$um) and large ($>=40$um) DRG neurons.

It is evident that whole DRGs are responsive to acute increases in neuromuscular activity in healthy rats (Molteni et al, 2004), and rats with SCI (Detloff et al, 2014) and sciatic nerve injury (Cobianchi et al, 2013). DRG gene expression changes are also reported after chronic exercise in rats with SCI (Detloff et al, 2014), pain (Chen et al, 2014) and DPN (Groover et al, 2013). However, such chronic changes have not been studied in healthy rats. Furthermore, neither acute nor chronic exercise effects on small and large DRG neurons in the healthy rat have been studied. Therefore, we studied chronic exercise in healthy rats to determine if DRG neuron mRNA changes occur, and if so, how these changes influence small and large DRG neuron gene expression.

Whether small and large DRG neurons respond differently to voluntary wheel verses forced treadmill exercise has not been previously examined. However, effects of treadmill (Chen et al, 2014; Cobianchi et al, 2013; Sun & Pan, 2014) and voluntary wheel (Detloff et al, 2014; Groover et al, 2013) exercise on whole DRG in non-healthy rodents have been investigated

separately, but not in comparison. Likewise, whole DRG gene expression was studied in healthy rats following acute voluntary wheel training (Molteni et al, 2004), but not treadmill training.

Due to the discrepancy of previously reported gene expression profiles in the DRG of healthy sedentary rats (please refer to introduction), our study will also aid in substantiating these findings. Furthermore, the gene expression results, in terms of exercise and technique, are novel.

Hypothesis

Hypothesis 1.1: *Gene expression of small and large DRG neurons show greater differences with 17 weeks compared to 1 week of forced treadmill exercise.*

Hypothesis 1.2: *Gene expression of small and large DRG cells of chronic treadmill exercised animals is different from that of voluntary wheel trained animals.*

Objectives

I.   To develop a protocol to visualize and isolate small and large DRG neurons while maintaining the integrity and maximizing the concentration of the sample RNA

II.   To establish qPCR parameters for the purpose of our study including determining the genes of interest

III.   To identify the relative changes in mRNA for each gene between small and large DRG neurons following treadmill and voluntary wheel training.

IV.   To evaluate if small and large DRG neurons exhibit the same differences in gene expression following long-term vs. short-term exercise training.

METHODS

For the acute exercise part of the study, 12 (6 control and 6 exercised; Rat IDs are TC and TE 48-53) healthy animals were run on the treadmill for 1 week. TC and TE were the two rat groups. TC indicates the sedentary control group, and TE indicates the treadmill exercised group. The training included a 3 day gradual "run-in" period to acclimate the rats to running at a pace of 22 meters/minute for 30 minutes at a 10 degree grade daily, for seven days.

Two different exercise protocols were utilized in the chronic exercise study, both treadmill (TE) and voluntary wheel (LW). Ten animals were used in the 17-week treadmill trained group (Rat IDs TE & TC 37-41; 5 exercised and 5 sedentary controls). Sixteen animals were used in the 16-week voluntary wheel trained group (Rat IDs LW & LC 9-16; 8 exercised and 8 sedentary controls). Animals were acclimated to treadmill running for 3 days (1 brief, low-intensity training session/day), then continued with one treadmill running session per day for 7 days with duration and intensity being progressively increased to a peak of 23 m/min at a 10 degree incline for 30 minutes. Speed and time of treadmill exercise were then gradually increased again to 27 m/min at a 10 degree incline for 60 minutes, twice per day, for 5 days per week by week 8. This level of intensity was maintained for the remaining 9 weeks of the study. Voluntary wheel trained animals had free access to their running wheels.

"Viable samples" refers to the DRG neurons of rats with enough LCM cells captured to allow RNA isolation, and those with sufficient quality and concentration of RNA for further processing (please refer to general methods for details). Hence, an individual rat can have usable RNA from a pool of small DRG neurons, yet have unusable RNA from a pool of large DRG neurons depending on experimental conditions and factors such as degradation.

RESULTS

Acute Exercise Study

Several other studies have used qPCR analysis to measure gene expression in whole DRG (Molteni et al 2004, Peeters et al, 2006), LCM of DRG cells (Harrington et al, 2011; Wang et al, 2007; Xu et al, 2007), and LCM of large DRG cells after spinal cord injury (Keeler et al, 2012), but none isolating and comparing small and large DRG cells specifically in healthy, endurance trained animals, that we know of. Hence, the precise method of LCM was a suitable technique for our purpose of separating single large and small DRG cells and analyzing picogram samples of RNA for differences in gene expression.

Control sedentary animals had an average start weight of 253g (ranged from 220-272g) and an average sacrifice weight of 291g (ranged from 250-322g). Treadmill trained animals had an average start weight of 251g (ranged from 218-274g) and an average sacrifice weight of 279g (ranged from 242-314g; all body weight changes were statistically significant using a t-test).

Based on preliminary testing, it was established that at least 400 laser captured cells were necessary (per size and per animal) to achieve a usable and meaningful RNA concentration. We generally captured many more cells than required to try and enhance the concentration and ensure our samples were viable.

Table 1-1: Summary of viable samples

	SMALL			LARGE		
Healthy Sedentary	LCM cell #'s	RIN	[pg/ul]	LCM cell #'s	RIN	[pg/ul]
TC48	735	7.6	738	619	7.2	814
TC49	778	7.5	369	510	7.2	770
TC50	795	7.4	473	592	7.3	847
TC52	864	7.6	697	1950	6.1	1285
TC53	860	6.9	547	615	7.4	497
Treadmill Exercised						
TE48	790	7.2	379	505	7.4	428
TE49	730	8	666	537	7	953
TE50	720	7.4	383	525	7.5	318
TE51	505	7.6	352			
TE52	792	7.4	350	761	7	542
TE53	1030	7.8	738	845	7.2	868

Key:
LCM – Laser Capture Microdissection
RIN – RNA integrity number (please refer to general methods for further information)
TC – Sedentary control animals
TE – Treadmill exercise animals

Animals TC51 small, TC & TE 51 large, were not included in the results due to their low amounts of recovered RNA. Hence, our analysis included 5 control and 6 exercised animals for small DRG neurons, and 5 control and 5 exercised animals for large DRG neurons.

All RNA samples were standardized at 318 pg/ul.

Differences in small and large DRG neuron gene expression in sedentary rats. The majority of genes studied significantly varied in their expression between small and large DRG neurons in sedentary animals (see Table 1-2). "Novel" refers to findings that were previously not reported.

Table 1-2: mRNA relative abundance in small vs. large DRG neurons – Ion channels

GENES	SEDENTARY	
	SMALL	LARGE
ION CHANNELS		
$Na_v1.2$	0.12 ± 0.01	0.10 ± 0.01
$Na_v1.3$	$*0.43 \pm 0.09$	0.13 ± 0.04
$Na_v1.6$	$*0.68 \pm 0.13$	2.27 ± 0.39
$Na_v1.7$	$*74.72 \pm 9.42$	41.31 ± 7.73
$Na_v1.8$	$*6034.86 \pm 799.00$	1574.41 ± 588.71
$Na_v1.9$	$*6086.17 \pm 1055.35$	713.44 ± 16.84
$K_v1.1$	$*0.70 \pm 0.12$	1.58 ± 0.57
$K_v1.2$	$*1.03 \pm 0.16$	2.03 ± 0.31
$K_{Ca}2.2$	$*0.70 \pm 0.16$	2.00 ± 0.77
$K_{Ca}2.3$	$*3.66 \pm 0.42$	2.63 ± 0.66
TRPV1	$*238.82 \pm 41.53$	38.15 ± 18.35

LEGEND:

Sedentary rats show differences in mRNA levels in small compared to large DRG cells with $p < 0.05$ (*). Levels of mRNAs in the L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Data are expressed as RQ means ± SD.

Ion channels. Ion channels Nav1.2, 1.3, 1.7, 1.8, 1.9, KCa 2.3 and TRPV1 are more

highly expressed in small compared to large DRG neurons. Ion channels Nav1.6, Kv1.1, 1.2 and

KCa 2.2 are more highly expressed in large compared to small DRG neurons.

Table 1-3: mRNA relative abundance in small vs. large DRG neurons – GABA and Adrenergic receptors

GENES	SEDENTARY	
	SMALL	LARGE
GABA & ADRENERGIC RECEPTORS		
$GABA_{A\alpha2}$	2.11 ± 0.52	2.51 ± 0.44
$GABA_{A\beta3}$	$*1.33 \pm 0.25$	1.95 ± 0.37
$GABA_{A\gamma2}$	5.33 ± 1.15	4.85 ± 1.17
$GABA_{B1}$	1.36 ± 0.22	1.22 ± 0.27
$GABA_{B2}$	3.77 ± 0.96	2.83 ± 0.70
$ADRA_{A1\alpha}$	$*0.04 \pm 0.02$	0.30 ± 0.07
$ADRA_{A1\delta}$	0.01 ± 0.01	0.01 ± 0.01

LEGEND:

Sedentary rats show differences in mRNA levels in small compared to large DRG cells with $p < 0.05$ (*). Levels of mRNAs in the L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Data are expressed as RQ means ± SD.

GABA and Adrenergic Receptors. Only two of the seven receptors examined, $GABA_{A\beta3}$ and $ADRA_{A1\alpha}$, were differentially expressed in small and large neurons, with both being more highly expressed in large relative to small DRG neurons.

Table 1-4: mRNA relative abundance in small vs. large DRG neurons – Glutamatergic receptors

GENES	SEDENTARY	
	SMALL	LARGE
GLUTAMATERGIC RECEPTORS		
NMDAR$_1$	*0.72 ± 0.24	0.40 ± 0.06
Glur2	*0.40 ± 0.06	0.57 ± 0.09
Glur3	*0.09 ± 0.01	0.20 ± 0.04

LEGEND:

Sedentary rats show differences in mRNA levels in small compared to large DRG cells with $p < 0.05$ (*). Levels of mRNAs in the L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Data are expressed as RQ means ± SD.

Glutamatergic Receptors. Glutamate receptors 2 and 3 are more highly expressed in large verses small neurons, whereas the NMDA1 receptor shows greater expression in small compared to large DRG neurons. Glur2 and 3 genes were previously found to be more highly expressed in small neurons.

Table 1-5: mRNA relative abundance in small vs. large DRG neurons − 5HT receptors

GENES	SEDENTARY	
	SMALL	LARGE
5HT RECEPTORS		
$5HT_{1A}$	*0.68 ± 0.22	0.29 ± 0.13
$5HT_{1B}$	*1.61 ± 0.46	3.36 ± 0.68
$5HT_{1D}$	*22.10 ± 2.82	71.38 ± 9.88
$5HT_{1F}$	*0.62 ± 0.12	0.37 ± 0.13
$5HT_{2A}$	*0.55 ± 0.08	0.41 ± 0.04
$5HT_{2C}$	*0.01 ± 0.00	0.04 ± 0.01
$5HT_{3A}$	*49.32 ± 6.78	30.19 ± 5.38
$5HT_7$	*0.60 ± 0.06	1.20 ± 0.26

LEGEND:

Sedentary rats show differences in mRNA levels in small compared to large DRG cells with $p < 0.05$ (*). Levels of mRNAs in the L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Data are expressed as RQ means ± SD.

5HT Receptors. mRNAs were present for all 8 5HT receptors studied. There was greater expression in small compared to large DRG neurons for: 5HT1A (novel), 5HT1F (novel), 5HT2A and 5HT3A (novel) receptors. Receptors 5HT1B, 5HT1D, 5HT2C (novel) and 5HT7 (novel) were more highly expressed in large compared to small DRG neurons. The most highly expressed receptor in small neurons was 5HT3A, and in large neurons was 5HT1D.

Table 1-6: mRNA relative abundance in small vs. large DRG neurons – Trks

	SEDENTARY	
GENES	*SMALL*	*LARGE*
TROPOMYOSIN RELATED KINASE RECEPTORS		
TrkA	*189.71 ± 52.10	107.13 ± 20.02
TrkB	0.62 ± 0.39	0.72 ± 0.12
TrkC	*1.27 ± 0.34	5.16 ± 1.00

LEGEND:

Sedentary rats show differences in mRNA levels in small compared to large DRG cells with $p < 0.05$ (*). Levels of mRNAs in the L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Data are expressed as RQ means ± SD.

Tropomyosin related kinase receptors. TrkA receptors were more highly expressed in small DRG neurons, and TrkC receptors were more highly expressed in large DRG neurons compared to small ones. TrkB receptor expression was not significantly different between small and large neurons, and was not highly expressed.

Table 1-7: mRNA relative abundance in small vs. large DRG neurons – Growth-related genes

GENES	SEDENTARY	
	SMALL	LARGE
GROWTH-RELATED		
NGF	0.48 ± 0.07	0.44 ± 0.18
BDNF	10.72 ± 2.83	7.57 ± 1.69
NT3	1.14 ± 1.39	1.22 ± 1.67
GAP43	*4.14 ± 0.85	1.85 ± 0.38
IGF1	1.38 ± 0.54	1.33 ± 0.63

LEGEND:

Sedentary rats show differences in mRNA levels in small compared to large DRG cells with $p < 0.05$ (*). Levels of mRNAs in the L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Data are expressed as RQ means ± SD.

Growth-related genes. GAP43 is more highly expressed in small compared to large DRG neurons. NGF, NT3, BDNF and IGF1 was not significantly different in gene expression between small and large DRG neurons (i.e. novel).

Table 1-8: mRNA relative abundance in small vs. large DRG neurons – Synaptic vesicle-related genes

	SEDENTARY	
GENES	*SMALL*	*LARGE*
SYNAPTIC VESICLE-RELATED		
STX1A	*6.86 ± 1.09	1.04 ± 0.13
STX1B	0.39 ± 0.07	0.53 ± 0.35
SYN1	*1.76 ± 0.26	1.35 ± 0.19
VGlut2	*2.09 ± 0.34	0.90 ± 0.09

LEGEND:

Sedentary rats show differences in mRNA levels in small compared to large DRG cells with $p < 0.05$ (*). Levels of mRNAs in the L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Data are expressed as RQ means ± SD.

Synaptic vesicle-related genes. STX1A, VGlut2 and Syn1 gene expression is higher in small compared to large DRG neurons (novel). STX1B was not significantly different between small and large neurons (novel).

Table 1-9: mRNA relative abundance in small vs. large DRG neurons – Pain-related genes

| | SEDENTARY | |
GENES	SMALL	LARGE
PAIN-RELATED		
SubP	*24.14 ± 1.87	3.45 ± 1.65
CGRP	*21.55 ± 5.34	5.90 ± 2.36
OPRM1	*5.64 ± 0.82	1.86 ± 0.76
OPRD1	*0.71 ± 0.09	3.05 ± 0.87
CNR1	*0.10 ± 0.02	0.30 ± 0.06

LEGEND:

Sedentary rats show differences in mRNA levels in small compared to large DRG cells with $p < 0.05$ (*). Levels of mRNAs in the L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Data are expressed as RQ means ± SD.

Pain Related Genes. Neuropeptides SubP and CGRP, as well as the mu-opioid receptor were more highly expressed in small compared to large DRG neurons. The delta-opioid receptor and the cannabinoid receptor were both more highly expressed in large relative to small DRG neurons.

Changes in DRG gene expression after 1 week of treadmill exercise. There were no statistically significant differences in gene expression in small or large DRG neurons after 1 week of treadmill exercise compared to sedentary animals (see appendix 6 for complete data). The only two genes that showed a trending ($p < .1$) difference in gene expression after acute exercise (Figure 1-1) were $5HT_{1A}$ in small neurons (increased by 56%), and $Na_v1.2$ in large neurons (decreased by 18%).

Figure 1-1: One week of treadmill running exercise effect on gene expression in small and large DRG neurons
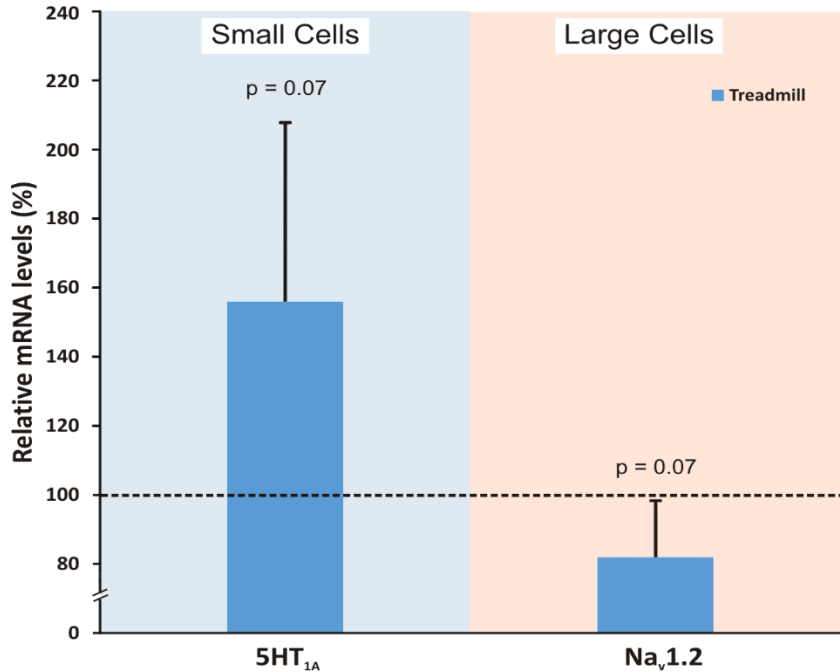
1 week of treadmill exercise shows no statistically significant (p<0.05) differences in mRNA levels in small or large DRG cells. mRNA levels in the small and large L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. mRNA levels with treadmill exercise are expressed as a percentage relative to the sedentary animals (sedentary represented by ------ at 100%). Standard deviation bars = SD for 5 animals per condition. 5HT1A and Nav1.2 each showed statistically trending differences when comparing the 1 week of exercise with the sedentary samples with p < 0.1 by 2-tailed t-test.

Chronic Exercise Study

Although none of the 32 genes tested in the acute exercise study significantly differed with exercise, we hypothesized that a longer exercise period would result in changes in gene expression. We already know that chronic adaptations in the neuromuscular system take longer than one week (Beaumont & Gardiner, 2003). Furthermore, chronic exercise results in changes in biophysical properties of motoneurons (Gardiner et al, 2006), and both acute and chronic exercise display gene expression changes at the motoneuron (Ferraiuolo et al, 2009; Woodrow et

al, 2013) and spinal cord (Gomez-Pinilla et al, 2001 & 2002; Ying et al, 2003; Perreau et al, 2005). Therefore we used 16 weeks of exercise, both voluntary (running wheels) and forced (motorized treadmill), to examine changes in small and large DRG gene expression. The result was 10 of the 27 genes tested exhibited statistically significant differences in the DRG with chronic increases in neuromuscular activity.

The body weights of animals at the beginning of the study ranged from 219g to 302g. The body weights of animals after exercise training ranged from 322g to 492g, and at the end of the study the control sedentary animals ranged in weight from 326g to 472g (all body weight changes were statistically significant using a t-test).

Table 1-10: Summary of viable samples

	SMALL			LARGE		
Sedentary	LCM cell #'s	RIN	[pg/ul]	LCM cell #'s	RIN	[pg/ul]
TC 37				736	7.8	284
TC 38	1045	8.2	448	657	7.6	329
TC 39	935	7.6	572	766	8.6	263
TC 40	1025	7.7	258			
LC 15	1005	7.6	433	652	7.6	553
LC 16				810	7.5	757
Treadmill						
TE 37	1055	7.7	238	844	4.8	753
TE 38	1095	7.6	271	757	7.4	223
TE 39	1262	7.6	514	1009	7.2	358
TE 40	1060	7.3	629	760	7.1	815
TE 41	1180	7.7	1142	1009	8.2	396
Wheel						
LW 9	1265	8.2	727	894	8	523
LW 10	1350	8.5	743	450	7.9	810
LW 12	1230	8.4	623	645	7.5	596
LW 13	1355	7.8	1167	925	7.2	1083
LW 14	1410	7.9	1159	453	7.4	586
LW 15	1075	7.8	984	805	7.7	954
LW 16	1305	8.3	1069	925	7.5	903

Key:
LCM – Laser Capture Microdissection
RIN – RNA integrity number (please refer to general methods for further information)
LC/TC = Sedentary control animals
LW = Voluntary wheel animals
TE = Treadmill animal

For the voluntary wheel study, usable mRNA estimates were obtained in 3 of 8 sedentary control animals and 7 of 8 exercised animals. For the treadmill study, there were only 4 out of the 5 control animals viable in the small DRG category, but all other samples were viable for analysis (ie. 5 treadmill trained animals). Because the control numbers were relatively lower, we combined the control animals from the wheel and treadmill studies as a common control group for both (ie. 4 controls for the small DRG category and 5 controls for the large DRG category).

All RNA samples were standardized at 223 pg/ul.

Figure 1-2: Voluntary wheel individual rat running distances
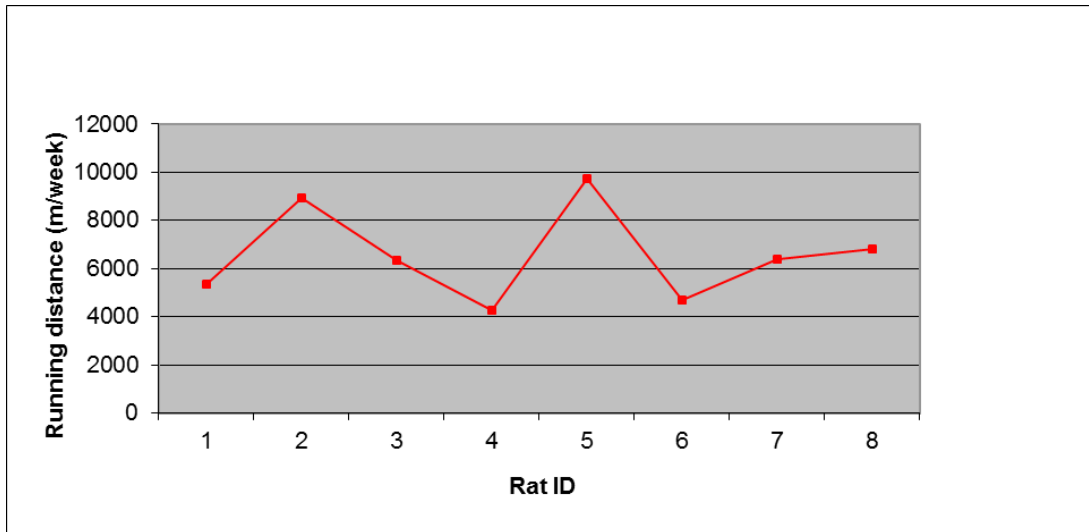
Legend:
Individual weekly running average distance over a 16 week period.
Kalan and Gardiner and Natasha Paddock supervised the voluntary wheel rats; Nadine Urbana and Natasha Paddock analyzed the data

The rat running the greatest distance, on average per week, was rat 5 at 9719 m/week,

and the lowest was rat 4 at 4263 m/week. Rat 2 had the highest running distance in one week at

15,473 m/week. Rats 1-2 and 4-7 decreased their average running distance per week from week

1 to week 16, while rats 3 and 8 increased their running distance.

Figure 1-3: Voluntary wheel group average running distances per week
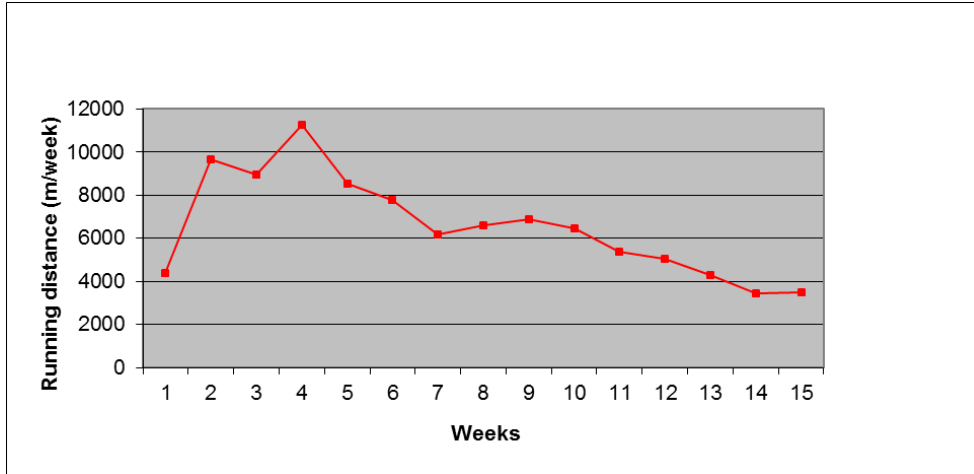
Legend:
Group average weekly running distance over a 16 week period (n=8).

The group's average weekly running distance was highest for week 4 at 11,252 m/week. The lowest average running distance for the group was week 15 at 3449 m/week. As you can see, on average, the group had a sharp increase in weekly running distance from week 1 to 4, and weekly averages gradually decreased to week 16.

Figure 1-4: Voluntary wheel total running distance per rat
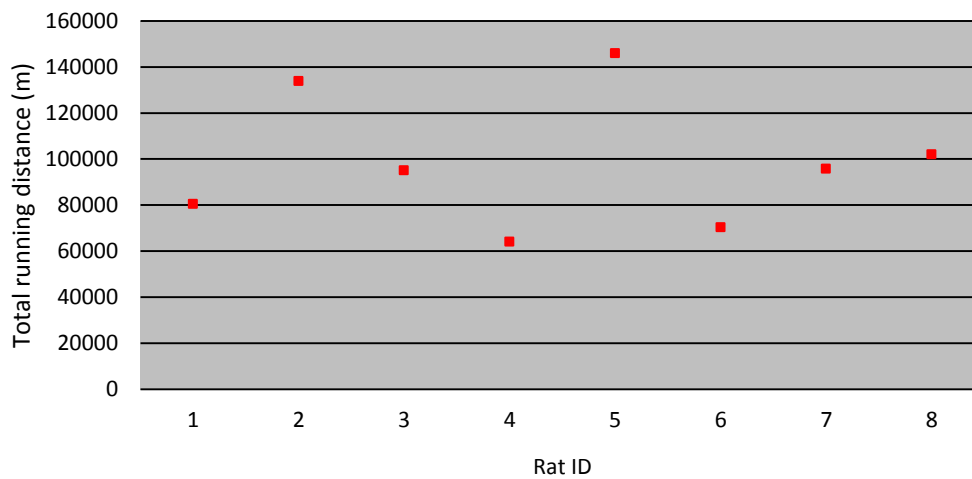
Legend:
Voluntary wheel individual rat total running distances over a 16 week period.

The average of the total distance ran by each rat in 16 weeks on a voluntary wheel was 98,340m. The sum of the average distances ran by the group per week in 16 weeks was the same. The highest distance runner over the training period was rat 5 at 145,789m. The lowest distance runner over the training period was rat 4 at 63,946m (for data please see appendix 5).

Therefore, voluntary wheel rats (98,340m) voluntarily ran less total distance on average compared to treadmill trained rats that were forced to run approximately 196,930m in 16weeks (i.e. 213,130m total in 17 weeks), when considering the duration of the study.

Changes in DRG gene expression after 16 weeks of treadmill exercise. There were several statistically significant differences in gene expression in both small and large DRG neurons after 16 weeks of treadmill exercise compared to sedentary animals (see appendix 6 for complete data). In small cells (Figure 1-5), 5HT1D was increased (229%) and 5HT1F was decreased (66%). In large cells (Figure 1-6), 5HT1A was decreased (71%), TrkC was decreased (25%), and SYN1 was decreased (22%).

Figure 1-5: Chronic treadmill and voluntary wheel exercise effect on gene expression in small DRG cells
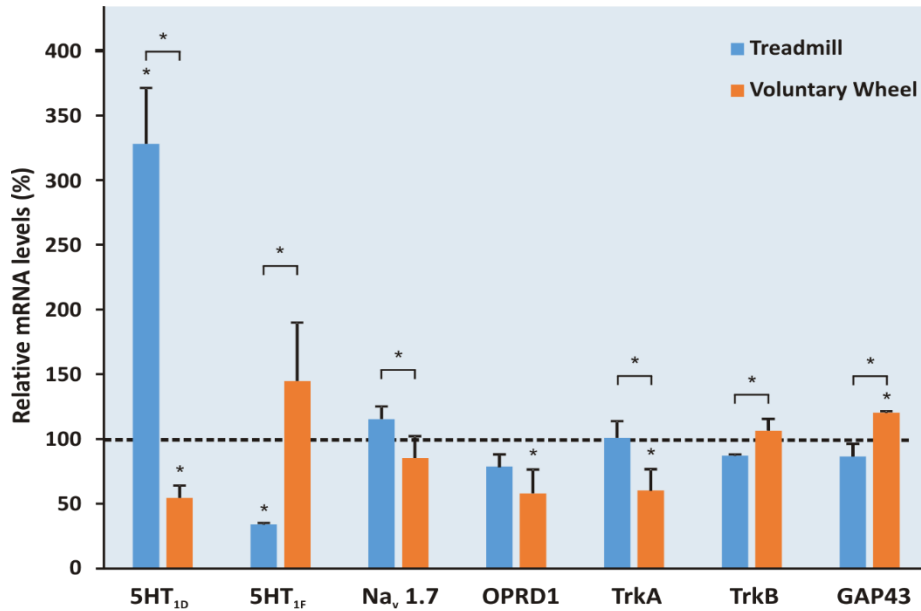
Legend:

16 weeks of treadmill and voluntary wheel exercise increases and decreases mRNA levels in small DRG cells. mRNA levels in the small L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Standard deviation bars = SD for 4-7 animals per condition. mRNA levels with exercise are expressed as a percentage relative to the sedentary animals (sedentary represented by ------ at 100%). Statistically significant differences between sedentary and exercised animals are indicated by an * above the bar. Line over bars with an * on top indicate statistically significant differences between treadmill and voluntary wheel exercised animals. $p < 0.05$ (*) by ANOVA and Tukey post-hoc test.

Changes in DRG gene expression after 16 weeks of voluntary wheel exercise. There were several statistically significant differences in gene expression in both small and large DRG neurons after 16 weeks of voluntary wheel exercise compared to sedentary animals (see appendix 6 for complete data). In small cells (Figure 1-5), 5HT1D was decreased (45%), OPRD1 was decreased (42%), TrkA was decreased (40%), and GAP43 was increased (21%). In large cells (Figure 1-6), 5HT3A was increased (34%), 5HT1D was decreased (41%), Nav1.6 was

decreased (18%), OPRD1 was decreased (32%), GAP43 was increased (49%), TrkA was

decreased (36%), TrkC was decreased (26%), and Syn1 was decreased (28%).

Figure 1-6: Chronic treadmill and voluntary wheel exercise effect on gene expression in large

DRG cells
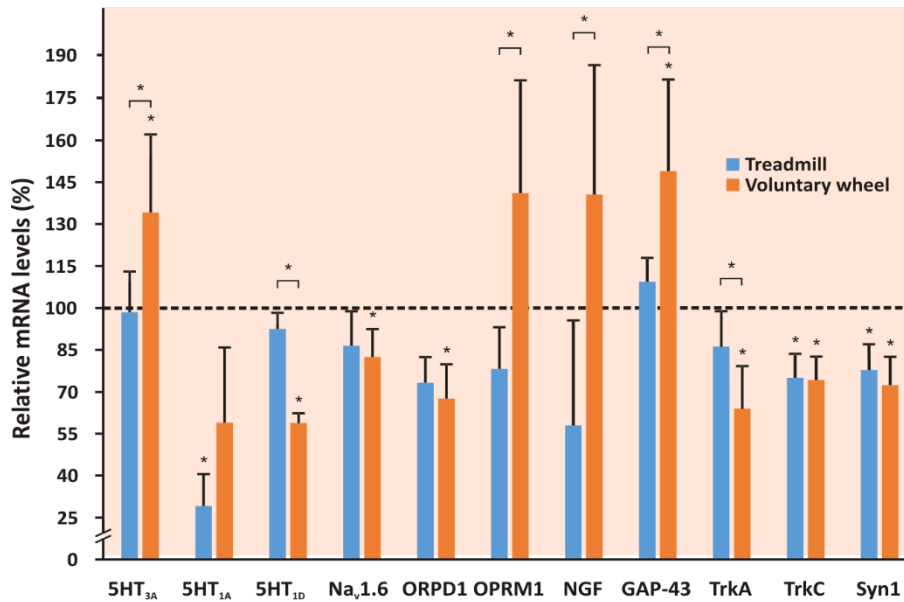
Legend:

16 weeks of treadmill and voluntary wheel exercise increases and decreases mRNA levels in large DRG cells. mRNA levels in the large L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Standard deviation bars = SD for 5-7 animals per condition. mRNA levels with exercise are expressed as a percentage relative to the sedentary animals (sedentary represented by ------ at 100%). Statistically significant differences between sedentary and exercised animals are indicated by an * above the bar. Line over bars with an * on top indicate statistically significant differences between treadmill and voluntary wheel exercised animals. $p < 0.05$ (*) by ANOVA and Tukey post-hoc test.

Changes in DRG gene expression after 16 weeks of forced treadmill compared to

voluntary wheel exercise. There were several statistically significant differences in gene

expression in both small and large DRG neurons after 16 weeks of treadmill exercise compared

to voluntary wheel exercised animals (see appendix 6 for complete data). In small cells (Figure

1-5), treadmill exercised animals showed greater expression than voluntary wheel exercised

animals for genes 5HT1D (274%), Nav1.7 (30%), and TrkA (41%). Treadmill exercised animals

showed lesser expression than voluntary wheel exercised animals for genes 5HT1F (111%), TrkB (19%) and GAP43 (34%). In large cells (Figure 1-6), treadmill exercised animals showed greater expression than voluntary wheel exercised animals for genes 5HT1D (34%) and TrkA (22%). Treadmill exercised animals showed lesser expression than voluntary wheel exercised animals for genes 5HT3A (36%), OPRM1 (63%), NGF (83%), and GAP43 (40%).

Both treadmill and voluntary wheel training exhibited differences in gene expression compared to sedentary rats for 5HT1D in small (Figure 1-5), and SYN1 and TRKC in large (Figure 1-6) cells.

In summary, this is the first demonstration of exercise causing differential changes in gene expression within small and large healthy DRG neurons. The majority of genes examined vary in their expression between small and large DRG neurons in sedentary animals. There were two main objectives to this study. Firstly, to evaluate if long-term trained small and large DRG neurons exhibit the same change in gene expression as short-term trained neurons. We found this was not the case. Chronic exercise, both voluntary and treadmill, alter the expression of several genes studied that does not correspond with the acute exercise lack of significant change. The second objective was to determine if forced treadmill trained animals vary in their gene expression compared to voluntary wheel trained animals. Several genes examined do indeed show differences in expression depending on whether the chronic increases in neuromuscular activity was induced by voluntary or forced endurance exercise.

DISCUSSION

Differences in Small and Large DRG Neuron Gene Expression in Sedentary Rats

Confirmations, discrepancies and novelties. All genes examined were previously reported in whole DRG. The majority of genes significantly vary in their expression in small compared to large DRG neurons in sedentary animals.

Ion channels. The current results revealed ion channels Nav1.2, 1.3, 1.7, 1.8, 1.9, KCa 2.3 and TRPV1 are more highly expressed in small compared to large DRG cells. Ion channels Nav1.6, Kv1.1, 1.2 and KCa 2.2 are more highly expressed in large compared to small DRG cells. It is evident that the majority of Nav channels are more abundant in small verses large DRG neurons, and hence are most likely important to nociceptive signal transmission. Our results agree with the findings of others (Ho & O'Leary, 2011; Theriault & Chahine, 2014; Wang et al, 2014; Jordt et al, 2000; Yu et al, 2008; Rasband et al, 2001; Wittmack et al, 2005; Fukuoka et al, 2008) except for our novel finding that Nav1.3 mRNA levels were greatest in small cells, whereas they were only reported in whole DRG previously. Nav1.3 has been previously shown in embryonic cells but only in small amounts in the adult (Rush et al, 2007; Dib-Hajj et al, 2009), unless injury or disease caused it to increase (Waxman et al, 1994; Samad et al, 2013). KCa2.3 showed higher expression in small cells in this study, whereas expression was shown to be comparable in cells of all sizes in previous studies (Mongan et al, 2005; Fukuoka et al, 2008). KCa2.2 was greater in large, whereas higher levels were previously found in small cells (Mongan et al, 2005). Kv1.1 mRNA levels were higher in large, whereas two conflicting previous reports show higher expression in small (Hao et al, 2013) and large (Rasband et al, 2001) cells. In contrast, the Kv channels examined were both predominantly found in large DRG neurons and hence most likely aid in proprioceptive signal transmission.

Nav1.8 and 1.9 were the most highly expressed of all genes examined suggesting their importance in excitability of small peripheral fibers.

   GABA and Adrenergic receptors. The current study revealed that only two of the seven receptor mRNAs examined, $GABA_{A\beta3}$ and $ADRA_{A1\alpha}$, were differentially expressed in small and large cells, with both being more highly expressed in large DRG cells. However, these results are in conflict to previous reports that $GABA_{A\beta3}$ was found in almost all cells in similar amounts (Coggeshall & Carlton, 1997), $ADRA_{A1\alpha}$ was most highly expressed in small cells (Trevisani et al, 2007), and the GABAB1 receptor was previously found at higher levels in small cells (Engle et al, 2012). The remaining receptors (GABAAα2, Aγ2, B2 and ADRAα1D) expression levels in DRG subtypes were previously not reported (i.e. novel findings) (Charles et al, 2001; Ma et al, 1993; Xie et al, 2001; Coggeshall & Carlton, 1997).

   Glutamatergic receptors. Our study demonstrated that glutamate receptors 2 and 3 are more highly expressed in large verses small cells, whereas the NMDA1 receptor mRNA shows greater expression in small compared to large DRG cells. The NMDA1 receptors results are consistent with previous findings (Sato et al, 1993; Liu et al, 1997; Marvizon et al, 2002). However, Glur2 and 3 genes were previously found to be more highly expressed in small cells (Sato et al, 1993; Coggeshall & Carlton, 1997; Carlton & Hargett, 2007; Willcockson & Valtschanoff, 2008).

   5HT receptors. There is discrepancy as to the serotonin (5HT) receptor subtypes detected in the DRG. All 15 subtypes have been investigated using various methods. 5HT receptors 1B, 1D, 2A, 3 and 4 have been previously confirmed in the lumbar DRG of rats (Pierce et al, 1996; Wu et al, 2001; Nicholson et al, 2003; Classey et al, 2010). However, there is conflicting evidence for other 5HT receptor subtypes present in rat lumbar whole DRGs. For instance,

Pierce et al (1996) performed PCR to detect mRNA for receptors 2C and 7, but did not detect receptors 1A, 1F, 2B or 5A. In another study utilizing RT-PCR, receptors 1A, 1F, 5A and 7 were present, but 2B and 2C were not. More recently in 2010, Classey et al. showed dense staining of the 1F receptor using immunohistochemistry. Nicholson et al (2003) performed in situ hybridization to identify 2B positive neurons present in all small, medium and large cells, but they were unable to detect 1A, 1E, 2C, 5A or 7 receptors. However, the 5HT7 receptor was previously found mainly in small to medium sized DRG cells (Doly et al, 2005; Cardenas et al, 1999). Furthermore, receptors 1B and 4 were more highly expressed in medium neurons, 1D and 3B in large neurons, and 2A in small neurons. These discrepancies amongst studies reveal the variability associated with gene expression results for 5HT receptors in lumbar whole DRGs in the rat. Therefore, four of these receptors are controversial in terms of detection in the DRG (i.e. 5HT1A, 1F, 2C, 7).

Our study showed mRNAs were present for all 8 5HT receptors studied. There was greater expression in small compared to large DRG cells for: 5HT1A (novel), 5HT1F (novel), 5HT2A and 5HT3A (novel) receptors. Receptors 5HT1B, 5HT1D, 5HT2C (novel) and 5HT7 (novel) were more highly expressed in large compared to small DRG cells. The most highly expressed receptor mRNA in small cells was 5HT3A, and in large cells was 5HT1D. These results are consistent with the positive findings of 5HT1D more greatly expressed in large cells, but inconsistent with the findings of 5HT1B most highly expressed in medium cells and 5HT7 mRNA levels higher in small to medium cells. The 5HT receptor mRNA most prominent in small DRG neurons (i.e. 1A, 1F, 2A, 3A) are more likely to play a role in nociception, whereas the ones more abundant in large cells (i.e. 1B, 1D, 2C, 7) may be more involved in proprioception.

Tropomyosin related kinase receptors. The current results exhibit the TrkA receptor more highly expressed in small DRGs, and TrkC receptor more highly expressed in large DRG cells compared to small ones. This is consistent with previous findings (Josephson et al, 2001; Ernsberger, 2009). TrkB receptor expression was not significantly different between small and large cells, and was not highly expressed. This was to be expected, and consistent with previous findings, as we did not collect medium DRG cells, which have been previously shown to predominately express TrkB receptors in rats (Mu et al, 1993; Wetmore & Olson, 1995; Ernsberger, 2009).

Growth-related genes. Our study demonstrated that GAP43 is more highly expressed in small compared to large DRG cells which agrees with previous work (Nacimiento et al, 1993). NGF, NT3, BDNF and IGF1 were not significantly different in gene expression between small and large DRG cells (new finding), which was surprising and in conflict to the findings of others which showed NGF and BDNF predominately found in small to medium DRG cells (Shu & Mendell, 1999; Ernfors et al, 1990), majority of NT3 in large DRG cells (Snider, 1994), and IGF1 mainly found in small cells (Craner et al, 2002).

Synaptic vesicle-related genes. The current mRNA results show STX1A, VGlut2 and Syn1 gene expression is higher in small compared to large DRG cells. STX1B was not significantly different between small and large cells. These are all new findings as STX1A, STX1B and SYN1 were previously found in whole DRG (Aguado et al, 1999; Molteni et al, 2004) but expression by cell sub-type was not determined. VGlut2 was reported in most all small, medium and large (Brumovsky et al, 2007; Landry et al, 2004) DRG cells, but predominate cell type expression was not known.

Pain-related genes. Our study showed that neuropeptides SubP and CGRP, as well as the mu-opioid receptor was more highly expressed in small compared to large DRG cells. This is consistent with previous work (Wang et al, 2010; Basbaum et al, 2009; Averill et al, 1995; Doughty et al, 1991; Scherrer et al, 2009; Coggeshall & Carlton, 1997). The delta-opioid receptor and the cannabinoid receptor were both more highly expressed in large relative to small DRG cells, whereas previously they were both reported more highly expressed in small cells (Wang et al, 2014; Scherrer et al, 2009; Coggeshall & Carlton, 1997).

Potential reasons for relative gene expression discrepancies in small and large DRGs. The reasons for these discrepancies may be attributed to the differences in species (i.e. rat vs. mice) and technique utilized (i.e. PCR vs. immunohistochemistry). Furthermore, we did not investigate medium sized DRG cells, so some receptors could potentially be more highly expressed in that population of DRG neurons (i.e. 5HT1B more highly expressed in Medium cells; Nicholson et al 2003). Therefore a limitation is that some genes shown here to be more highly expressed in small or large cells, could potentially have higher expression in medium cells. However, we were interested in the nociception and proprioception pathways which are primarily the smallest and largest fibers/cells respectively.

Effects of Acute vs Chronic Exercise on DRG neurons

It is evident that whole DRG are responsive to acute increases in neuromuscular activity in healthy (Molteni et al, 2004) and nerve-injured (Detloff et al, 2014; Cobianchi et al, 2013) rats. DRG gene expression changes have also been reported after chronic exercise in abnormal conditions (Detloff et al, 2014; Chen et al, 2014; Groover et al, 2013). However, such chronic changes have not been studied in healthy rats. Furthermore, neither acute nor chronic exercise

effects on small and large DRG neurons in the healthy rat have been studied. Therefore, we studied chronic exercise in healthy rats to determine if DRG neuron changes occur, and if so, the extent to which these changes are gene and/or neuron size dependent.

Acute exercise effects on the DRG

Healthy model. The only study to date supporting the DRG neuron response to acute exercise in the healthy, non-injured rat is by Molteni et al (2004). They showed that 3 and 7 days of voluntary wheel exercise increased mRNA levels of BDNF, GAP43, SYNI and NT3 in whole DRG, as well as induced neurite outgrowth in culture, suggesting exercise increased neuron growth capacity by increasing neurotrophin signalling. The Molteni study provides evidence that the DRG is responsive, and can potentially adapt, to increases in activity. In contrast, our study using qPCR of small and large laser-captured DRG cells, showed no statistically significant difference in gene expression of these particular genes in either small or large DRG neurons in response to 7 days of treadmill exercise in healthy rats.

There are several potential reasons for the discrepancy between studies. First of all, the second part of study 1 revealed some major differences in gene expression in small and large DRG neurons between treadmill and voluntary wheel trained animals. Therefore, the difference in exercise modality of voluntary wheel used by Molteni et al and treadmill used in the current study could account for discrepancies. Also, with the current technique of LCM to capture large and small neurons, the samples were "pure" cells. In contrast, whole DRG samples will include other confounding substances included in the DRG such as satellite glial cells, which also contain BDNF and NT3 (Zhou et al, 1999; Liu et al, 2003) for example. Another influencing

factor for discrepancy could be size of cells collected. We excluded medium sized neurons from our study which may account for the different levels of expression in the whole DRG.

A recent study by Sheahan et al, 2015 showed that 1 week of voluntary wheel exercise, limited to 2 hours of exercise per night with control animals having access to a locked wheel, in healthy mice showed no change in behavioral (von Frey, Hargreaves, hot plate and cold plantar assay tests), electrophysiological (whole small DRG cell recordings in culture) or skin fiber (hindpaw staining for CGRP/peptidergic epidermal fibers) testing. Therefore, voluntary wheel exercise did not alter the nociception threshold, small sensory neuron excitability or skin innervation in these mice (Sheahan et al, 2015). Although our study used the treadmill exercise paradigm, rats instead of mice, focused on nociceptive and proprioceptive neurons verses mainly nociceptive responses, the lack of functional implications implied by the absence of change in gene expression in small and large DRG cells, is consistent with our findings.

Injured model. There are limited studies supporting the DRG neuron response to acute exercise in the injured rat. Keeler et al (2012) showed that passive cycling after SCI resulted in no change in mRNA levels of BDNF, NT3, NT4, GDNF and their receptors in large laser-captured lumbar DRG neurons of the rat. However, changes were noted at the motoneuron and whole spinal cord levels. In another study investigating SCI, rats exercised on automated wheels for two and seven weeks, beginning five days post-SCI injury, preventing the SCI-induced decrease in GDNF protein levels in the dorsal horn of the spinal cord, as well as in cervical whole DRGs (Detloff et al, 2014). The attenuation was accompanied by a decrease in tactile allodynia, indicating that exercise prevents the onset of neuropathic pain associated with SCI (Detloff et al, 2014). Additionally, five days of treadmill training after sciatic nerve injury

resulted in a decrease in BDNF, NGF and GDNF, and no change in NT3 mRNA levels in whole

lumbar DRGs of the rat, compared to injured rats without exercise (Cobianchi et al, 2013). These

gene expression results were accompanied by a decreased neuropathic pain response, further

supporting the hypoalgesic effect of treadmill training after peripheral nerve lesion (Cobianchi et

al, 2013). One week of treadmill training after peripheral nerve injury in rats, was accompanied

by a decrease in immune-reactivity of GAP43, NGF and BDNF in DRG neurons, as well as

reduced hyperalgesia (Lopez-Alvarez et al, 2015). One week of voluntary wheel training, after

sciatic nerve gap and consequent transplant of nerve graft, caused an increase in GAP43 and

Syn1 mRNA in rat whole DRG ipsilateral to the reconstructed peripheral nerve gap (Haastert et

al, 2008). Conversely, no transplant resulted in no effect of exercise on DRG gene expression

(Haastert et al, 2008).

Based on the preceding studies, it seems that acute treadmill exercise after peripheral

nerve injury results in down-regulation in neurotrophins and GAP43 in rat whole DRG.

However, with sciatic nerve gap followed by voluntary wheel exercise, there was no change in

GAP43 or SYN1 expression in whole DRG, whereas, nerve transplantation to fill the gap

followed by voluntary wheel exercise resulted in GAP43 and Syn1 up-regulation in whole DRG.

However, in spinal cord injury studies compared to peripheral nerve injury studies, there is no

change in neurotrophin expression in large and whole DRG neurons after voluntary wheel or

passive cycling exercise. Hence, the type of exercise and type of injury model used, result in

different DRG gene expression responses.

In the current study, healthy rats, after treadmill exercise, showed no statistically

significant changes in small and large DRG cells in the genes described above, suggesting lack

of functional implication on nociception or proprioception related to these particular genes. The only injury model study that is coherent with these results is Keeler et al, 2012 who showed no change in large DRG gene expression of BDNF, NT3, TrkB and TrkC after SCI and acute passive cycling exercise. Therefore, whole DRG gene expression may give different results in changes in gene expression compared to single cells in DRG sub-types. Furthermore, treadmill exercise following nerve injury compared to voluntary wheel exercise showed differing responses in whole DRG gene expression suggesting exercise type is relevant to the functional outcome. Another consideration is that nerve injury may be required to induce certain gene expression changes in small and large DRG cells exposed to acute treadmill exercise.

Chronic exercise effects on the DRG

Healthy model. Information regarding chronic exercise effects on DRG gene expression in healthy animals is limited. To our knowledge, there are no studies in rats to date. Groover et al, 2013 used 12 weeks of voluntary wheel exercise to study NGF and BDNF protein levels in healthy mice whole DRG. They found no change in protein levels after exercise. This is consistent with our study that showed no change in NGF or BDNF mRNA levels in small or large DRG cells in 16 week voluntary wheel or treadmill exercised, compared to healthy sedentary rats. However, NGF was significantly higher in voluntary wheel trained relative to treadmill trained rats in large DRG cells.

Injured/Diseased model. Groover et al, 2013 also studied mice fed high fat diets to induce pre-diabetes and associated painful neuropathy. Twelve weeks of voluntary wheel exercise improved neuropathic symptoms including normalized: neurotrophin levels, epidermal fiber density, mechanical allodynia and visceral hyperalgesia. There was an increase in NGF

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protein in the DRG, muscle, and skin in high-fat fed sedentary mice, which was significantly

normalized by exercise in the skin only. Hence pre-diabetes induction was required to perceive

the beneficial effect of exercise in the skin but not in the DRG. Chen et al, 2014 used the

skin/muscle incision and retraction surgery as a rat model of inducing postoperative pain.

Sedentary animals exhibited prolonged mechanical hypersensitivity, as well as an up-regulation

of SubP protein in whole DRG. Four weeks of forced treadmill running shortened the recovery

time from persistent pain, in addition to reversing the increase of SubP. Three weeks of treadmill

exercise prior to heart injury resulted in increased CGRP mRNA in rat whole DRG, producing a

cardio-protective effect (Sun & Pan, 2014). Four weeks of passive cycling exercise after SCI

produced no change in BDNF, TrkB, NT3 and TrkC mRNA levels in rat large DRGs (Keeler et

al, 2012).

In light of the genes examined above, our study showed no significant change in SubP,

CGRP, TrkB or NT3 gene expression in small or large DRG cells after 16 weeks of voluntary

wheel or treadmill trained rats. However, TrkB was more highly expressed in small cells of

voluntary wheel exercised compared to treadmill trained rats. Therefore, injury and/or pain, as

well as prolonged exercise periods, may be necessary to induce a change in small and large DRG

gene expression of certain genes after exercise.

Acute vs chronic exercise effect differences are also apparent in other cell types. Molteni

et al (2002) studied the effect of 3, 7 and 28 days of voluntary wheel exercise on gene expression

in the hippocampus and found that specific molecular pathways are activated after different

periods of exercise. Alaei et al (2007) found that 30 days of treadmill training resulted in

increased learning and memory whereas 8 days of exercise did not. Similarly, Di Loreto et al

(2014) showed that 4 months of treadmill training caused beneficial actions on preserving brain senescence, while 2 months of exercise had the opposite negative effect in brain cortex.

In summary, it is evident that small and large DRG neurons are responsive to increases in neuromuscular activity. The differences in gene expression between acute and chronic exercise show that the more plentiful long-term changes are not due to the last exercise bout, but are in fact most likely steady-state changes. Therefore, there may not be a short-term response in DRG neurons to increase the production of certain genes in response to the start of a treadmill exercise program (ie. a new physiological stressor; although this response may vary with voluntary wheel exercise). However, with potential adaptation to chronic exercise, there may be a new physiological role or need for increased or decreased expression of particular genes. Neurons that show a change in activity exhibit adaptations that are most likely preceded by changes in gene expression. For example, three days of treadmill exercise prior to induced heart injury resulted in no change in synthesis or release of CGRP, whereas 3 weeks of exercise resulted in increased CGRP gene expression in the DRG as well as release in the blood and heart of rats, producing a cardio-protective effect (Sun & Pan, 2014). This study also demonstrates differing effects of acute verses chronic exercise. Keeler et al (2012) showed increased GDNF family receptor α1 (GFRa1) mRNA levels in large DRG neurons of rats subjected to SCI followed by 31 days of exercise, which may correspond to enhanced functional recovery. However, ten days of exercise post-injury did not show any significant change in mRNA levels. Therefore, once the physical activity is not considered "new" to the body anymore, a new steady state is established. Overall, it seems that acute exercise changes do not give us a window into the chronic changes that occur when exercise is continued for a longer period of time.

Forced Treadmill Compared to Voluntary Wheel Chronic Exercise Effects on the DRG

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The difference between the effects of treadmill verses wheel running exercise have not been extensively studied. Whether small and large DRG neurons respond differently to voluntary wheel verses forced treadmill exercise has not been previously examined. The effects of treadmill (Chen et al, 2014; Cobianchi et al, 2013; Sun & Pan, 2014) and voluntary wheel (Detloff et al, 2014; Groover et al, 2013) exercise on whole DRG in non-healthy rodents have only been investigated in separate studies. Likewise, whole DRG gene expression was studied in healthy rats following voluntary wheel training (Molteni et al, 2004), but not treadmill training.

The only study we are aware of examining the effects of chronic voluntary wheel exercise on DRG gene expression is by Groover et al (2013), where they investigated NGF and BDNF levels. The only chronic forced exercise study to look at either one of these genes in the DRG is by Keeler et al (2012), who studied BDNF expression after SCI and subsequent cycling training. However, the former study included mice exercising for 12 weeks and tested whole DRG samples, whereas the latter had rats exercising for only 4 weeks and only used large DRG cells. No changes were noted in BDNF levels in either study.

Our study showed several differences in gene expression when comparing chronic treadmill to voluntary wheel exercise. Genes that were more highly expressed in voluntary wheel compared to treadmill exercise include: 5HT1F, 5HT3A, TrkB, GAP43, NGF, and OPRM1. Genes that were more highly expressed in treadmill compared to voluntary wheel exercise include: 5HT1D, Nav1.7, and TrkA. Our study also showed that DRG neurons were more responsive to chronic voluntary wheel exercise compared to treadmill exercise, as evidenced by more differences in gene expression relative to sedentary controls. 5HT1D and 5HT1F in small cells, and 5HT1A in large cells, seem to be more responsive to chronic treadmill exercise; whereas OPRD1, TrkA, and GAP43 in small cells, and 5HT3A, 5HT1D, Nav1.6, OPRD1,

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GAP43, TrkA, TrkC and Syn1 are more highly expressed after voluntary wheel exercise. Furthermore, in some treadmill trained animals neuron gene expression is not different compared to healthy sedentary animals, but the same gene's expression is either up- or down-regulated with voluntary wheel exercise, and vice versa. The only gene that responded differently between the two forms of exercise compared to sedentary, was 5HT1D in small cells (ie. significantly lower mRNA compared to healthy sedentary with voluntary wheel training, but significantly higher expression compared to healthy sedentary with treadmill training).

It was very interesting to find the differences in gene expression between treadmill and wheel running. The main difference between these two types of training seems to be the total distance ran during the training period. With treadmill training resulting in significantly more distance ran compared to voluntary wheel exercise, it may be that treadmill exercise is focused on shorter duration exercise bouts of higher intensity, while wheel exercise involves longer duration bouts at a lower intensity. Thus, some genes are sensitive to increased intensity of training while others are more affected by duration of increased activity.

In addition, there is the obvious difference that treadmill training can be considered forced behavior which may enhance stress (Conner et al, 2014, Alaei et al, 2007, Ploughman et al, 2005, Yanagita et al, 2007; Moraska et al, 2000) leading to anxiety (McDevitt & Neumaier, 2011), while running on a wheel is voluntary. However, others believe treadmill running is actually more similar to how humans exercise (Di Loreto et al, 2014) compared to voluntary exercise. Grissom & Bhatnagar (2009) describes habituation to repeated stress where treadmill trained animals adapt to the stress and levels return to normal after a period of time of daily training. Leasure & Jones (2008) showed that 8 weeks of voluntary wheel and treadmill training

have differing effects on rat brain and behaviour, stressing the point that the inherent difference of both types of exercise needs to be considered when prescribing treatment. This is also evident in our results. In light of the varying and sometimes opposite effect of exercise type on gene expression in small and large DRG cells, the type of exercise prescribed should be based on the patients/athletes status (i.e. pain, disease, performance, rehabilitation, etc.). Sheahan et al (2015) showed no change in nociception, inflammatory or neuropathic pain after 1-4 weeks of voluntary wheel exercise in mice, compared to the previously described suppression of pain reported after treadmill exercise (Stagg et al, 2011; Cobianchi et al, 2010 & 2013). Therefore, voluntary verses forced exercise may also result in different functional implications (i.e. exercise analgesia), that may be correlated to the differences in gene expression in small and large DRG cells. There does not seem to be a trend for certain sub-classes of genes to be more or less affected by one type of training compared to the other. However, both small and large cell gene expression seem to be regulated similarly. For instance, exercise caused differences in mRNA levels of 5HT1D, OPRD1, TrkA and GAP43 in both small and large cells. These differences in gene expression showed similar patterns of change between voluntary and forced exercise.

Exercise Effects in Small and Large DRG cells

One week of treadmill exercise showed no statistically significant differences in gene expression compared to sedentary animals in any of the genes examined in either small or large DRG cells. There was a trending up-regulation of the $5HT_{1A}$ receptor in small cells (by 56%; p=0.07) and down-regulation of $Na_v1.2$ in large cells (by 18%; p=0.07). Although other genes were either up- or down-regulated by large percentages compared to sedentary, no other p-values were less than 0.1. However, the acute treadmill exercise study contributed to the studies feasibility (i.e. LCM technique in general, LCM cell numbers required, RNA integrity achieved,

etc.) as well as served as a control for the subsequent chronic exercise studies. The indication of exercise causing differences in gene expression of small vs large in acute studies (i.e. both ours using treadmill and Molteni et al (2004) using voluntary wheel where significant changes in whole DRG gene expression were found), warranted further research into long-term changes in gene expression of small and large cells. Based on both the acute and chronic exercise studies, we learned that changes in chronic exercise are not due to the last exercise session, but rather, they are steady-state and therefore most likely reflect protein and functional changes (i.e. not a reflection of what happened in the last day of training session). Therefore, study 1 established a baseline for the effect of exercise on gene expression in small and large DRG cells. A limitation is that medium cells were not collected, and hence, it is possible that those neurons could show changes in gene expression with acute or chronic exercise (i.e. especially for genes that are more highly expressed there in sedentary animals).

It is likely that changes in mRNA levels are reliable indicators of changes in gene expression in our studies (please see intro pgs. for more detail). It is understood however, that in all cases mRNA levels may not reflect protein levels, and hence the functional significance in the sensory system may be undetermined. Further work would have to be completed to see if protein mirrors mRNA in these models. However, a further consideration is that protein is not necessarily located in the soma and can be translated in and transported to other areas of the neuron. A molecular study could include semi-quantitative immunofluorescence where density of a particular protein is measured.

The mechanism by which exercise alters gene expression in DRG neurons is not clear, but may involve changes in electrical activity and molecular signalling caused by exercise (Gardiner, 2006). Pain research suggests that electrical activity and molecular signalling in the

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DRG activate second messenger cascades that result in production of molecules that are then transported to both the central and peripheral terminals where they are released out of the terminal or inserted into the membrane to regulate synaptic strength and excitability (Woolf & Costigan, 1999). The response of transcriptional changes at the DRG is caused by an increase in activity at the DRG as well as retrograde transport of signal molecules from the periphery to the DRG, such as NGF/TrkA (Mantyh et al, 2011). Hence, the potential increased synthesis of 5HT1A, 5HT1D and 5HT1F receptors, Nav1.2 and GAP43 could lead to the anterograde transport of these molecules from the DRG to the spinal cord and peripheral terminal membranes. Furthermore, exercise also causes an increase in action potential generation and altered molecular signalling and therefore may resemble a similar mechanism of gene expression alterations to that of pain. For instance, exercise causes an increase in neurotrophin levels in muscle (Ying et al, 2003), DRG (Molteni et al, 2004), spinal cord (Gomez-Pinilla et al, 2001) and brain (Neeper et al, 1995). An increase in BDNF/TrkB signalling within the DRG neuron for example, could cause 2nd messenger signalling to increase the phosphorylation of CREB and alter gene expression (Lesser et al, 1997). It was previously shown that exercise causes an increase in CREB expression in the spinal cord (Gomez-Pinilla et al, 2002).

With the assumption of protein synthesis of the newly expressed genes, they can be transported and act (i.e. change in ion conductance) at DRG neuron terminals and other cell types including spinal neurons. A change in biophysical properties within these latter cells and consequent cell function shows that exercise causing a change in gene expression could be the mechanistic basis for these activity-related adaptations (Gardiner, 2006).

Chronic exercise regulates gene expression in small and large DRGs. Some general observations were that there were more significant changes after voluntary wheel exercise (i.e. compared to treadmill exercise) in both small and large DRG cells; more significant change in large (i.e. compared to small) cells; and more decreases in gene expression evident with exercise (i.e. vs. increases).

Our 16 week voluntary wheel and treadmill exercise studies showed that the most prominent and statistically significant changes in gene expression were for the 5HT1A, 5HT1D and 5HT1F receptors, and GAP43, and hence the genes of focus for the discussion of gene changes.

5HT1A, 1D & 1F receptors are regulated in DRG cells after chronic exercise. This is the first study to show a change in 5HT1A receptor gene expression in DRG neurons after exercise. Although the receptor message was increased by 56% in small cells after one week of voluntary wheel exercise, this difference was not statistically significant (p=0.07). In contrast, a down-regulation of the 5HT1A receptor was evident in large cells after chronic treadmill exercise. Since 5HT1A showed change only with treadmill, and not voluntary wheel exercise, it suggests that this receptor may require higher intensity forced activity, to induce change.

This is the first study to show a change in 5HT1D receptor gene expression in DRG neurons after exercise. The up-regulation of the 5HT1D receptor was evident in small cells after chronic treadmill exercise. The down-regulation of the receptor was evident in both small and large cells after chronic voluntary wheel exercise.

This is the first study to show a change in 5HT1F receptor gene expression in DRG neurons after exercise. The down-regulation of the 5HT1F receptor was evident in small cells

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after chronic treadmill exercise, therefore this receptor may require higher intensity forced activity, to induce change.

There are two main responses to 5HT receptor activation, including facilitation or inhibition. A facilitatory response can be mediated by depolarization, increased firing, synaptic transmission and neurotransmitter release, while an inhibitory response elicits the opposite effects (Fozard, 1984). 5HT is increased in the brain with exercise, and therefore there could be increased descending 5HT input to the spinal cord. With more access to 5HT, and higher/lower receptor numbers available to be activated, the 5HT receptors present on afferent terminals could modulate either of these responses (i.e. excite or inhibit). Therefore, 5HT can act at both central and peripheral terminals to regulate pain, and therefore, is a good potential candidate for mechanism of action in exercise analgesia. Based on the current results, the 5HT1D receptor is likely involved in exercise analgesia associated with chronic treadmill exercise, whereas the 5HT1A receptor most likely contributes to exercise analgesia related to acute treadmill exercise. Chronic voluntary wheel exercise seems to have the opposite effect, as it resulted in a decrease in the 5HT1D receptor in small cells. How acute voluntary exercise effects the expression of these 5HT receptors in healthy small DRG cells is not known (please see pgs. 116-117 for further explanation). The present work shows a molecular basis, other than opioid receptors, for sensory neuron processing of exercise analgesia.

5HT can also regulate responses in large afferents. Our results showed a decrease in 5HT1A and 1D receptors in large DRG cells after chronic treadmill and voluntary wheel exercise respectively. Hence, these receptor changes may be implicated pre-synaptically to reduce pre-synaptic inhibition of neurotransmitter release, leading to increased excitability of the post-

synaptic neuron (i.e. decrease in MSR and increase in afferent transmission), and therefore could contribute to enhanced motor output (i.e. improved motor coordination).

5HT has been well implicated in pain and in a limited number of studies in exercise analgesia. Branch chain amino acids (BCAAs) circulating in the blood, enter the brain on the same carrier as tryptophan (i.e. 5HT precursor). Since exercise caused an increase in BCAAs consumed by skeletal muscles, less BCAAs were available to enter the brain and more tryptophan could enter (Newsholme et al, 1995), thereby possible increasing 5HT synthesis. Also, exercise caused an increase in fatty acids in the blood, also leading to more tryptophan entering the brain. Other studies measured and confirmed that brain 5HT levels were increased with exercise in the rat, and 5HT metabolites, indicative of 5HT levels, were increased in humans (Aan het rot et al, 2009; Dunn et al, 1996). This would presumably enhance the amount of 5HT available to be released and act at the spinal cord, thereby increasing the inhibitory effect on sensory transmission. For example, 5HT produced in brainstem nuclei projecting to the dorsal spinal cord activated certain 5HT receptors that acted to suppress incoming noxious input, thereby inhibiting pain transmission to the spinal cord (Alhaider et al, 1991; Basbaum et al, 1984). Alternatively, inhibiting 5HT synthesis decreased the antinociceptive response to exercise (Mozzardo-Martins et al, 2010). Furthermore, a low expression of the 5HT transporter (5HTT) in humans is related to a higher threshold to thermal pain and hypoalgesia (Lindstedt et al., 2011).

Serotonin was found in small DRGs (Bojkowski et al, 1983), coming from various potential sources including 5HT fibers present in dorsal roots (Di Carlo, 1983), the DRG cell body (Kai Kai et al, 1989), mast cells in peripheral nerve and the DRG (Kai Kai & Keen, 1985), and circulating 5HT which can access the DRG cell membrane (Artigas et al, 1985). Amounts of

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5HT were similar in DRG and the brain (van Steenwinckel et al, 2009). Hence, 5HT may have a physiological role on the soma of DRG cells, with small DRG neurons being most sensitive to 5HT (Molokanova et al, 1995). Therefore, it is plausible that 5HT receptors that are found in the cell bodies actually function locally. For instance, activation of the receptor in DRG cells leads to decreased excitability in only 4% of A-type cells and 39% of C-type cells; and causes increased excitability in 82% of A-type cells and 41% of C-type cells (Todorovic & Anderson, 1992). Therefore, C-type cells respond with hyperpolarization more than A-type cells, and A-type cells have a greater depolarization response than C-type cells. Furthermore, activation of 5HT1A receptors in C-type cells inhibits calcium currents and decreases the action potential duration (Cardenas et al, 1997). Visceral sensory neurons were depolarized by 5HT (Higashi & Nishi, 1982). Hence, since each 5HT receptor was found in small and large DRG cells in varying relative amounts in the current study, it is possible they could be present and function within the neuron.

The same receptor can have different actions at the peripheral terminal, axon, cell body and central terminal. For instance, 5HT modulates transmission of nociceptive signals, and 5HT released in the periphery leads to hyperalgesia (Suftka et al, 1991; Fields et al, 1991). In peripheral terminals, 5HT acts through 5HT1A receptors, which activate the cAMP 2nd messenger system, causing hyperalgesia (Taiwo & Levine, 1992; Taiwo et al, 1992). Activation of peripherally acting 5HT1A receptors resulted in a reduction of chronic pain (Andrews & O'Neill, 2011). 5HT1A, 1D and 1F agonist produce antinociception in the rat formalin test, causing a reduction in inflammatory pain (Granados-Soto et al, 2010). Another study found that 5HT1D activation was not involved in peripheral nociception in mice (Nascimento et al, 2011). Hence, the type of pain involved may activate different receptors. Research related to 5HT

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receptors in peripheral terminals that are not related to pain/C-fibers is sparse. The majority of the 5 HT response is in C-fibers. Therefore, any changes that were noted in 5HT receptors in large DRGs either have an unknown or negative effect at the peripheral terminal.

5HT1A receptors are also found in primary afferent terminals in the spinal cord (Laporte et al, 1995). Although 5HT1A receptors are mainly found in LI-III of the dorsal horn (Laporte et al, 1995) and are widely accepted as playing a role in anti-nociception, they are also located in small amounts in the ventral horn (Laporte et al, 1995). The large diameter neurons indicate myelinated afferent fibres (i.e. Aα and Aβ), which terminate and form synapses in both the dorsal and ventral horn. Metabotropic 5HT receptors can act as pre-synaptic autoreceptors or heteroreceptors (Feuerstein, 2008). There is no conclusive evidence that 5HT is released from primary afferents. However, 5HT released within DRG neurons could be transported to the peripheral or central terminals to be released, and/or 5HT could be released from 5HT afferent neurons, and function as a neurotransmitter (Di Carlo 1983; Kai-Kai & Keen, 1985; Ruda & Gobel, 1980). The majority of the 5HT response in primary afferent terminals is in C-fibers (Hagashi et al, 1982; Stansfeld et al, 1982; Fock & Mense, 1976), where the activated 5HT receptor, 1A for example, functions by decreasing glutamate release and causing anti-nociception (i.e. pre-synaptic inhibition (PSI)) (Yoshimura & Furue, 2006). Hence, activation of this pre-synaptic receptor attenuates post-synaptic neuronal firing, and thus, spinal transmission of sensory messages. Additionally, the $5HT_{1A}$ receptor responds to 5HT released from the descending serotonergic pathway by inhibiting spinal nociception in intact and neuropathic animals (Liu et al, 2002). A study examining acute spinal pain, found no effect of receptors 1D and 1F, but the 1A receptor was the main anti-nociceptive mediator according to rat LII neuron recordings (Jeong et al, 2012).

The presence and role of these receptors in A-fiber pre-synaptic terminals are less clear. There is only low labelling for 5HT1A and 1D receptors in the ventral horn (Marlier et al, 1991; Yoshimura & Furue, 2006). The majority are located at LI-III of the dorsal horn (Meuser et al, 2002). There is very little known about the proprioceptive afferent terminal response to 5HT in the spinal cord, especially related to the role of 5HT receptors in the ventral horn, as 5HT modulation predominates in pain and temperature afferents.

Gosgnach et al (2000) showed that PSI causes a decrease in the monosynaptic EPSP during fictive locomotion in the cat. The indication of MSR depression was thought to prevent undesirable motor output. However, the group 1a pre-synaptic mechanisms (i.e. receptors) causing the decrease in neurotransmitter release was not alluded to. Dougherty et al (2005) used a 5HT1A/7 agonist that reduced monosynaptic potentials in dorsal horn interneurons that are post-synaptic to group II muscle afferent terminals. They determined the post-synaptic receptors on the dorsal horn interneurons, rather than the pre-synaptic receptors, probably had the biggest effect on 5HT produced inhibition. However, 5HT3 receptors were examined and found to operate pre-synaptically to decrease transmission. It was suggested that 5HT1A and other metabotropic 5HT receptors may also act pre-synaptically to modulate 5HTs action on group II afferents. Changes in the polysynaptic pathway from the afferent, to dorsal horn interneuron, to commissural interneuron and finally alpha-motoneuron, influences the motor response. Garcia-Ramirez et al (2014) investigated myelinated skin and muscle afferents monosynaptic to dorsal horn interneurons, and the effect that 5HT has on primary afferent depolarization (PAD). They found that 5HT, activating metabotropic receptors, regulated sensory transmission. However, since DRPs were greatly reduced (i.e. decrease in PSI/increase afferent transmission), it was concluded that interneurons (IN) were the main site of 5HT receptor activation, leading to the

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decreased monosynaptic transmission. Still, the decrease noted in extracellular field potentials (EFP) could potentially be related to decreased synaptic efficacy (i.e. decreased neurotransmitter release) of the afferents, associated with an attenuated post-synaptic receptor response (i.e. glutamate receptor).

Perrier & Cotel (2015) reviewed the direct monosynaptic connection between large afferent terminals and alpha-motoneurons in the ventral horn, and how it is affected by high intensity exercise eliciting prolonged release of 5HT. They summarized that 5HT1A receptors located on motoneurons (i.e. post-synaptically) inhibit excitability of the motoneuron, inhibit locomotion, and increase central fatigue, which are opposite responses compared to a low level of 5HT release. The pre-synaptic 5HT receptors that could influence this response have not been studied.

Neither of the preceding articles included exercise. In our study, 5HT receptors are regulated differently at the DRG by chronic treadmill vs voluntary wheel exercise, and acute vs. chronic treadmill exercise. Therefore, it is possible that exercise may cause changes (i.e. regulate DRG gene expression) that alter the activity of these receptors on the afferent terminal. For instance, decreased expression of the 5HT1A receptor in large DRG cells after chronic treadmill exercise could indicate less receptor availability at the proprioceptive afferent terminals in dorsal and ventral horns, leading to less PSI and enhanced neurotransmitter release to the dorsal horn interneurons or motoneurons that are involved in the motor response (i.e. less fatigue and enhanced locomotion performance via increased motoneuron excitability).

5HT1D and 5HT1F receptors have mainly been studied in relation to anti-migraine effects in the TGN; however, they are present in the DRG, and therefore can possibly be involved in other types of pain processing as well. 5HT1D receptors were mainly found on

primary afferents in LI and LII of the spinal cord, with few projecting to LIV, where they can regulate neurotransmitter release (Potrebic et al, 2003). 5HT1D agonists working at TGNs relieve migraine pain (Potrebic et al, 2003). Furthermore, both gamma-motoneurons and a large fraction of proprioceptive DRG cells are labelled by the 5HT1D receptor in mice (Enjin et al, 2012). In 5HT1D knockout mice, the MSR is depressed, accompanied by improved motor coordination skills (i.e. decreased excitability of proprioceptive circuits) (Enjin et al, 2012). The authors concluded that 5HT1D was involved in the proprioceptive circuit's ability to process accurate sensory information, and determined the receptor was acting post-synaptically. In another study, 5HT1D receptors located in the ventral spinal cord modulated motor output by decreasing monosynaptic reflex transmission in spinal rats (Honda et al, 2004). However, it was not determined in that study, whether the receptors were acting pre- or post-synaptically of the A-fiber terminal.

Greater or less activation of the 5HT1D receptors on primary afferent terminals could mean increased or decreased neurotransmitter release, respectively. An up-regulation in small cells with treadmill exercise could mean a greater anti-nociceptive effect and hence play a role in exercise analgesia, with the opposite response occurring in small cells after voluntary wheel exercise. A down-regulation in large cells could mean greater depression of the MSR and thus improved coordination during certain movements after voluntary wheel exercise. The opposite results of gene expression changes after treadmill verses voluntary wheel exercise in small DRG cells, as well as the difference in effect of voluntary wheel exercise compared to treadmill exercise on 5HT1D receptor gene expression in large cells, displays differential regulation of nociceptive and proprioceptive sensory inputs and subsequent motor outputs by voluntary and

treadmill exercise. Therefore, the potential effects on exercise analgesia in particular, seems to indicate that the analgesia response may be dictated by type of exercise performed.

To our knowledge, there is no prior information regarding 5HT1F receptors involvement in the exercise response. There is also very limited research available related to 5HT1F receptors in general, compared to other 5HT receptors and their actions in the nervous system. Most studies have focused on 5HT1F receptors anti-migraine effects. For example, Classey 2010 and Agosti 2007 show that 5HT1F receptors acting at the TGN have an anti-migraine effect, but they also suggest that due to the receptor's presence in DRG cells, that they could be targeted for treatment of other types of pain as well. Hence, in a pain model, activation of the peripheral 5HT1F receptor results in anti-nociception. Our results show that regulation of the 5HT1F receptor is decreased in small cells after chronic treadmill exercise, indicating its potential role in altering nociceptive transmission.

Centrally, 5HT1F receptors seem to be confined to primary afferent terminals, and play an anti-nociceptive role in the spinal cord dorsal horn (Millan, 2002; Castro et al, 1997). Similar to other 5HT1 receptors, 1F acts to inhibit pain transmission via activation of Gi coupled proteins that inhibit adenylate cyclase, thereby inhibiting intracellular cAMP (Boess & Martin 1994; Millan, 2002). The majority of the 5HT response in primary afferent terminals is in C-fibers (Hagashi et al, 1982; Stansfeld et al, 1982; Fock & Mense, 1976), where the activated 5HT receptor appears to function by decreasing glutamate release (i.e. PSI), causing anti-nociception (Yoshimura & Furue, 2006). Hence, activation of the pre-synaptic 5HT receptor, influences post-synaptic neuronal firing, and thus, spinal transmission of sensory messages. In large muscle afferents, the 5HT1F receptor was suggested to act pre-synaptically to decrease EPSPs after SCI, resulting in reflex inhibition and spasm control (Murray et al, 2011). Similarly, other studies

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indicate that 5HT receptors may act pre-synaptically in large afferents, influencing

neurotransmitter release and PSI (Perrier & Cotel, 2015; Dougherty et al, 2005). The particular

pre-synaptic metabotropic receptors involved have not been identified.

In summary, 5HT1F receptors are present on peripheral and central terminals, and at the

DRG soma. In pain models, activation of the receptor results in anti-nociception at the periphery

and centrally. A decrease in the 5HT1F receptor in small cells, suggests that chronic treadmill

exercise attenuates the anti-nociceptive response.

We found that after chronic endurance exercise there is a significant difference in

5HT1A, 1D and 1F receptor mRNAs in DRG neurons compared to sedentary animals, which

may translate into a change in the levels of these receptors transported both peripherally and

centrally. Centrally, the receptors can be inserted on the primary afferent terminal and can affect

pre-synaptic regulation of neurotransmitter release (i.e. modulate synaptic efficacy).

Peripherally, receptors can also be trafficked to the terminal and act to modulate excitability of

the DRG neuron, and influence the neurogenic responses to pain. Therefore, our results

demonstrate changes in gene expression in 5HT receptors that may result in changes in

transmission of nociceptive and proprioceptive afferent information with exercise, and this

response varies based on type and duration of exercise.

GAP43 is up-regulated in small and large DRG cells after chronic voluntary wheel

exercise. This is the first study to show a change in GAP43 gene expression in DRG neurons

after chronic exercise. The up-regulation was evident in both small and large cells after voluntary

wheel exercise. A study by Molteni et al (2004) showed higher mRNA levels in whole DRG

GAP43 gene expression, and enhanced neurite outgrowth, after 3 and 7 days of voluntary wheel

exercise compared to sedentary rats. This study concluded that the increase in GAP43 was exercise dependent, and was related to the increased rate of axonal outgrowth after peripheral nerve crush. We did not perform any such functional outcome measures. However, the large increase in GAP43, in large cells especially, indicate that the growth/regenerative potential could be even greater with chronic exercise. It would be interesting to study the neurite outgrowth of small vs. large cells, to see if the axon elongation corresponds to the level of increase in GAP43, and if the two fibre types respond differently functionally, to increases in GAP43. Furthermore, since our one week treadmill training study did not reveal any significant differences in GAP43 mRNA levels, nor did 16 weeks of treadmill training, it suggests that voluntary wheel training in particular, may influence axonal growth.

It has also been shown that GAP43 is involved in synaptic function and plasticity (Routtenberg et al, 2000; Skene, 1989). Phosphorylation of GAP43 by PKC is associated with the neurotransmitter release in the hippocampus resulting in learning and LTP (Routtenberg et al, 2000), as well as with nerve terminal sprouting (Benowitz & Routtenberg, 1997). Therefore, it is plausible that a similar phenomenon occurs at the afferent central terminal. An increase in GAP43 in the DRG neuron could imply enhanced synaptic efficacy via increased neurotransmitter release to the post-synaptic neuron upon depolarization. This effect would presumably be greater in the proprioceptors, as exercise had the greatest effect on GAP43 mRNA levels in that neuron population.

GAP43 also acts as an osmosensory protein (i.e. mechanosensation), playing a transducer role, and reducing the threshold for downstream signalling by increasing the intracellular calcium concentration in DRG neurons (Caprini et al, 2003). Hence, an increase in GAP43 mRNA at the DRG could result in higher levels of the protein at the peripheral terminal, and

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enhanced signal transmission. In particular, since the increase in GAP43 mRNAs was evident in small and large cells after chronic voluntary wheel exercise, we would expect regulation of mechanosensation in both nociceptors and proprioceptors.

GAP43 mRNA expression in the brain changes with altered afferent signal activity (Rosskothen-Kuhl & Illing, 2014). This is consistent with the GAP43 up-regulation in DRG cells due to chronic voluntary wheel exercise noted here.

Conclusions

In conclusion, this study revealed several new findings. Expression in small or large DRG cells was compared for several genes in sedentary animals. Higher expression in small compared to large cells was found for: Nav1.3, 5HT1A, 5HT1F, 5HT3A, VGlut2, STX1A and Syn1. Higher mRNA levels in large relative to small cells was revealed for: 5HT2C and 5HT7. No significant difference in expression between small and large cells was determined for: GABAAα2, GABAAγ2, GABAB2, ADRAα1D, STX1B, NGF, NT3, BDNF and IGF1. Discrepancies in previous findings of small compared to large DRG neuron gene expression levels were also addressed.

Exercise can induce changes in small and large DRG cell gene expression. We compared DRG gene expression after acute and chronic treadmill exercise, and after voluntary wheel and treadmill chronic exercise. Our hypothesis that gene expression of small and large DRG neurons show greater differences with 17 weeks compared to 1 week of forced treadmill exercise was confirmed. Our second hypothesis that gene expression in small and large DRG cells of chronic treadmill exercised animals is different from that of voluntary wheel trained animals was true for

certain genes. Large cells showed a greater number of genes that differed in expression due to exercise compared to small cells, and thus potentially are more responsive/sensitive to increases in neuromuscular activity. There were no apparent patterns that specific classes of genes were preferentially targeted by increased activity, although multiple 5HT and Trk receptors were influenced. These results produce the first information of its kind that we are aware of. The impact of exercise on gene expression in the absence of some other intervention such as injury or disease, provides necessary baseline data for future studies in this field.

We conclude from these results that small and large DRG neurons have the ability to change in response to exercise. The response of small and large DRG neurons is based on whether the exercise is forced or voluntary. Furthermore, the data suggest a new alternative to exercise analgesia mechanisms including 5HT receptors 1D with chronic treadmill exercise, and perhaps 5HT1A with acute treadmill exercise. Regulation of these 5HT receptors with chronic exercise may also play a role in attenuating central fatigue and enhancing motor output. Lastly, chronic voluntary wheel exercise may be advantageous to axonal growth, nociceptive and proprioceptive signal transmission from the periphery, and synaptic efficacy as evidenced by the regulation of GAP43 at the DRG.

Study 2: The Effect of Chronic Increases in Neuromuscular Activity on Gene

Expression in Small and Large Dorsal Root Ganglion Neurons: Diabetic Rat

ABSTRACT

Dorsal root ganglion (DRG) neurons are responsive to altered neuromuscular activity and play a role in diabetic peripheral neuropathy (DPN). Previous research has shown that chronic treadmill exercise training can delay the onset of DPN. Understanding the effect of exercise on gene expression in small and large DRG neurons, in diabetic rats, may provide insight into mechanisms involved in changes to sensory processing associated with thermal hypoalgesia. We present evidence that small and large DRG neurons are differentially affected by diabetes and exercise.

We examined gene expression in samples of small and large cells of the L4/L5 lumbar DRG and their specific responses after diabetes and exercise to identify potential molecular processes involved in activity-dependent changes. DRGs were collected 4 hours after the last exercise session, and RNA was isolated from samples of large and small DRG cells following laser capture microdissection. Relative mRNA levels were determined using real-time polymerase chain reaction experiments.

STZ-induced diabetic rats received sedentary treatment or 15 weeks of voluntary wheel exercise. Behavioural testing of thermal latency response was performed on all animals. Control animals were healthy and sedentary. Gene expression analysis focused on ion channels, serotonergic receptors, tyrosine kinase receptors, growth-related genes and neuropeptides because of their potential roles in nociception and proprioception.

Diabetes with and without voluntary wheel exercise did not significantly alter the expression of mRNA for any genes examined in large DRG neuron samples. Small DRG neurons from diabetic sedentary animals contained higher 5HT1F and lower TrkB mRNA levels than those from healthy sedentary animals. In small DRG neurons, there was a decrease in

CGRP mRNA in diabetic voluntary wheel exercised animals. Five weeks of diabetes resulted in prolonged withdrawal latencies in both sedentary and exercised rats. 8-11 weeks of diabetes resulted in a longer withdrawal latency in diabetic sedentary rats, but not exercised rats, compared to healthy sedentary rats. Voluntary wheel exercise lowered the level of 5HT1F transcript and prevented the decrease in TrkB receptors caused by diabetes. These genetic changes may be correlated with the prevention/reversal of thermal hypoalgesia associated with DPN.

Our results demonstrate that exercise affects expression of genes involved in activity-dependent neural plasticity differentially in small and large DRG neurons, and that these changes vary with DPN. For the genes tested, small neurons were more affected than large neurons in diabetic animals. The results suggest potential new mechanisms for the delay in diabetic peripheral neuropathy associated with exercise.

PURPOSE & RATIONALE

This thesis comprises an investigation into the differences in gene expression of small and large DRG neurons, in diabetic sedentary rats, and in response to exercise. The novel approach of isolating small and large DRG neurons via LCM has not been used to investigate sensory signalling changes associated with DPN.

The first study showing the depletion of cutaneous nerves and neuropeptides in patients with diabetic neuropathy was released in 1989 (Levy et al., 1989). Abnormal innervation was examined using the immunocytochemical study of skin biopsies. Later, in 1996, Kennedy et al. further quantified the epidermal nerves in patients with diabetic neuropathy via a neuron tracing system, revealing a decrease in the number and length of epidermal nerve fibers.

Christianson et al (2003) were the first group to show iENF loss in skin in a mouse model of diabetes. The Wright laboratory proceeded to investigate the effect of exercise on diabetic neuropathy following these key findings. Namely, Kluding et al (2012) completed the first study examining the effects of a 10-week aerobic and resistance exercise program on nerve function in patients with DPN. Exercise resulted in decreased pain and neuropathic symptoms, and improved iENF branching of neuropathic and cutaneous nerve fibers. Subsequently, Groover et al (2013) used mice fed high fat diets to induce pre-diabetes and associated painful neuropathy. Twelve weeks of voluntary wheel exercise improved neuropathic symptoms including normalized mechanical allodynia and visceral hyperalgesia, neurotrophin levels, and epidermal fiber density. Furthermore, it was shown in human studies that long-term aerobic exercise can delay and even prevent the onset of DPN (Balducci et al, 2006), and forced exercise has been shown to delay the onset of neuropathic pain sometimes associated with DPN (Shankarappa et al, 2011). Although

our study does not involve neuropathic pain or forced exercise, but rather thermal hypoalgesia and voluntary wheel exercise, these studies provide evidence that DPN is affected by exercise.

Therefore, our study addressed the effect of voluntary chronic exercise on thermal hypoalgesia in STZ rats, and whether or not gene expression changes in small and large DRG neurons provide mechanistic insight into DPN progression.

Hypothesis

Hypothesis 2.1: *Diabetic rats that exercise will experience a delay in the onset of thermal hypoalgesia.*

Hypothesis 2.2: *There will be differences in DRG gene expression in response to exercise, providing evidence of molecular mechanisms to support hypothesis 2.1.*

Objectives

I. To determine if diabetic rats that exercise experience a delay in onset, or prevention of thermal hypoalgesia.

II. To determine if functionally significant gene expression in small and large DRG neurons is modified in diabetes, and how this regulation is affected by exercise.

METHODS

32 male rats were obtained from Charles River in a weight range of 201-225 ± 10g (approx. 9 weeks old). They were given a single intraperitoneal (IP) injection of *Streptozotocin* (STZ; 90mg/kg; please see appendix 1 for details) 9 days after arrival (270-325 g). STZ is an antibiotic that destroys pancreatic beta-cells, and is therefore used experimentally to induce type 1 diabetes. The animals were considered diabetic by 2 consecutive weekly blood glucose (BG) measurements of 19 mmol/L or higher. BG levels were monitored throughout the experiment (i.e. every 2-4 weeks). Rats were not treated with insulin. Darrel Smith and the RO Burrell staff completed this work.

5 days post-STZ injection, rats were placed in one of the 3 following groups: 1) STZ voluntary wheel animals (exercised for 14-15 weeks) 2) STZ sedentary (with no access to a running wheel) 3) Healthy sedentary. The rats were housed in pairs prior to going in the running wheels and singly after. The rats in the running wheel cages had 2 water bottles, while the others had an automatic watering system. Water bottles were changed every day. All rats were fed Labdiet 5001 on arrival. Marc Morissette supervised the voluntary wheel running.

When the exercise protocol was finished, animals were sacrificed 4 hours after removal from the cage. All animals were decapitated without anaesthesia. The rats were placed in a DecapiCone (from Braintree Scientific) prior to decapitation. Immediately following decapitation, DRGs were removed and flash frozen.

Behavioral testing

Behavioral testing is used to investigate nerve function. The Hargreaves behavioural test (Hargreaves et al, 1988) determines thermal hypoalgesia (i.e. indicative of DPN) by 3 months

post-STZ injection. This test is used to determine the animal's response to heat by selectively activating C fibers. The test is performed on the plantar surface of the paw by a focused, radiant heat light source (70°C) to the top of the glass surface creating an intense spot on the paw. The rat reacts with a paw withdrawal response from the radiant thermal stimulus. An extended paw withdrawal response latency indicates C fiber transmission dysfunction.

For our experiments, the machine was set to automatically stop after the lamp had been on for 20 seconds. The temperature rises at approximately 1°C each second, so that in 20 seconds it would go from 30 to 50 °C. The glass temperature is normally around 30°C. The details of the machine are described in Dirig et al, 1997. Darrell Smith performed this work.

Figure 2-1: Hargreaves Machine (UARDG, Dept. of Anesthesiology, University of California, San Diego 92103-0818).
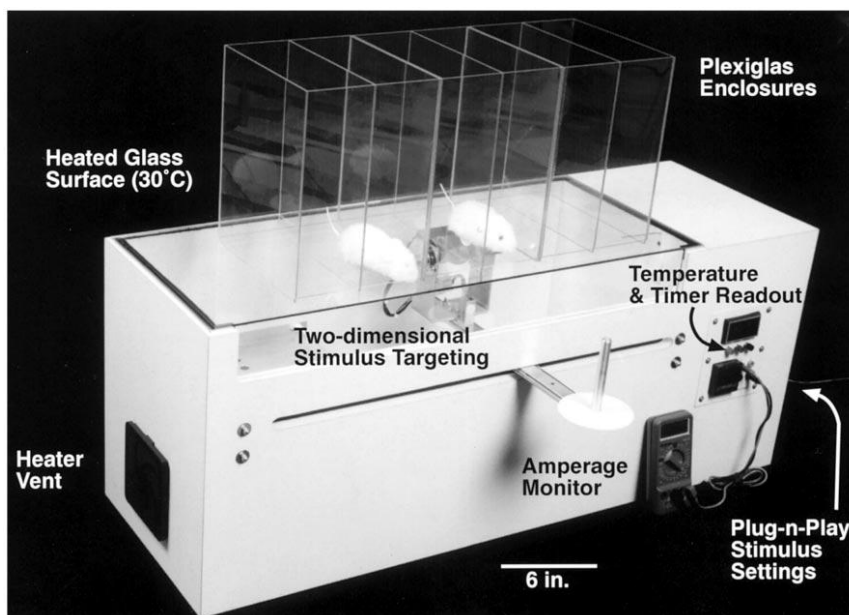
RESULTS

Table 2-1: STZ dosage, weight and blood glucose levels (Diabetic rats with access to running wheel; n=12)

Rat ID	STZ Dose	WEIGHT (g)		BLOOD GLUCOSE (mmol/L)	
		Pre-Exercise	Post-Exercise	Pre-Exercise	11 weeks
963-2	0.027	309.5	406.3	25.3	38.4
967-1	0.026	299.9	388.4	30.9	28.4
968-1	0.025	277.2	328.8	27.7	31.3
969-1	0.026	301.9	383.5	27.9	32.2
970-1	0.026	316.1	386.2	33.4	38.8
971-1	0.026	298.4	382.2	28	29.3
973-1	0.027	348.7	394.1	32.8	>40
974-1	0.027	294.6	381.6	30.9	36.7
979-1	0.025	294.1	376.4	27.8	35
980-1	0.026	314.6	366.8	26.5	29.6
981-1	0.028	308.1	365.3	27.9	29.2
982-1	0.026	301	391	38.4	31.1
Average ± SD		305.3 ± 17.2	379.2 ± 19.4	29.79 ± 3.7	32.7 ± 3.8

Table 2-2: STZ dosage, weight and blood glucose levels (Diabetic rats with no access to running wheel; n=10)

Rat ID	STZ Dose	WEIGHT (g)		BLOOD GLUCOSE (mmol/L)	
		Start	12 weeks	Start	11 weeks
968-2	0.027	314.3	416.7	31.3	30.4
969-2	0.027	322.9	461.2	28.7	31.4
971-2	0.026	302.4	383.3	28.6	33.3
974-2	0.028	305.7	401.5	24.9	30.7
975-2	0.025	319.2	412.7	30.3	25.6
976-2	0.025	312.5	410.7	24.7	37.6
979-2	0.026	320.4	434.5	30.7	30.6
980-2	0.027	332.6	425.2	30.1	29.7
981-2	0.027	295	368.1	25.3	32.2
982-2	0.026	296.6	392.8	30.9	34
Average ± SD		312.2 ± 12.2	410.7 ± 26.5	28.6 ± 2.6	31.6 ± 3.1

From the above Tables 2-1 and 2-2, it is evident that all blood glucose levels were sustained above 19 mmol/L, and therefore rats were considered diabetic.

Table 2-3: **Weight and blood glucose levels** (Non-diabetic and no access to running wheel; n=7)

Rat ID	WEIGHT (g)		BLOOD GLUCOSE (mmol/L)	
	Start	Sacrifice	Start	Sacrifice
965-1	345.4	677.9	10.2	8.6
965-2	358.5	807.2	9.8	8.4
966-1	322.6	632.3	9.3	8.8
966-2	339.2	805.1	7.7	9.1
977-2	347.8	620.3	8.8	8.9
978-1	379.1	887.4	9.9	11.6
978-2	313.3	647.9	7.5	9.4
Average ± SD	343.7 ± 21.9	725.4 ± 105.9	9.0 ± 1.1	9.3 ± 1.1

According to Table 2-3, all blood glucose levels in healthy sedentary rats were below 19 mmol/L and therefore non-diabetic.

"Pre-exercise" or "Start" as stated in the tables above, indicate between four to seven days after STZ injection, prior to identified animals entering the running wheel cage. From this time point to 11 weeks diabetic or sacrifice (ie. anywhere from 14 to 15 weeks later), the animals had average weight gains of 74g (STZ voluntary wheel), 99g (STZ sedentary) and 382g (Healthy sedentary; all body weight changes were statistically significant using a t-test, while blood glucose level changes were not).

Figure 2-1: Average withdrawal latencies using the Hargreaves test
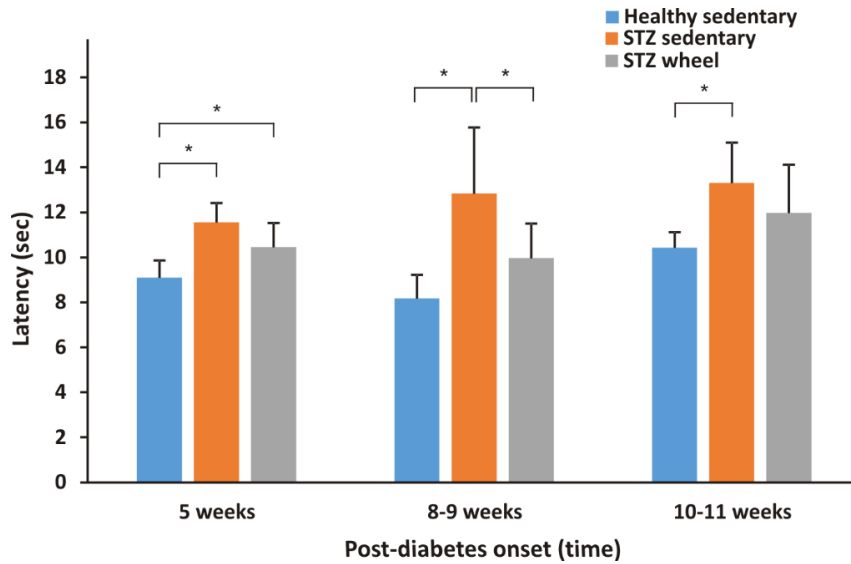
Legend:

Thermal latency testing in healthy sedentary controls and STZ-diabetic rats treated with sedentary or voluntary wheel exercise between weeks 5 and 11 of diabetes. Differences in average withdrawal latency times (s) were as follows: STZ sedentary showed statistically significant differences compared to healthy sedentary at 5, 8-9 and 10-11 weeks; STZ wheel showed statistically significant differences compared to healthy sedentary at 5 weeks; STZ wheel showed statistically significant differences compared to STZ sedentary at 8-9 weeks; with $p < 0.05$ (*) by ANOVA and Tukey post-hoc test. Data are group mean ± SD.

STZ sedentary rats displayed significantly longer withdrawal latencies compared to healthy sedentary rats at all 3 time points. STZ rats that exercised for 12 weeks on a voluntary wheel demonstrated a significantly longer withdrawal latency compared to healthy sedentary rats at 5 weeks, but no change was evident at weeks 8-9 and 10-11. At 8-9 weeks only, STZ exercised rats showed significantly shorter withdrawal latencies compared to STZ sedentary rats.

These results (Figure 2-1) show that by 5 weeks both sedentary and exercised diabetic rats exhibit a prolonged withdrawal latency, compared to healthy sedentary animals, indicative of thermal hypoalgesia. This response is consistent up to 11 weeks in the diabetic sedentary rats. However, STZ rats with daily voluntary wheel exercise did not significantly differ in withdrawal latency from healthy sedentary rats at 8-9 and 10-11 weeks, indicating that thermal hypoalgesia

was not present in these rats. Furthermore, at 8-9 weeks, the withdrawal latency of diabetic exercised rats was significantly less compared to diabetic sedentary rats. Thermal hypoalgesia indicates diabetic peripheral neuropathy. These behavioural results suggest that by 8 weeks of voluntary wheel exercise in STZ rats, thermal hypoalgesia is reversed/prevented (i.e. in contrast to 5 weeks), and this effect may be maintained up to 11 weeks. This effect is not as strong/clear at 10-11 weeks compared to 8-9 weeks (i.e. STZ wheel rats do not differ in withdrawal latency compared to healthy sedentary rats indicating lack of thermal hypoalgesia in these animals, even though STZ wheel rats also do not differ in latency compared to STZ sedentary rats at 10-11 weeks), possibly due to the general decrease in exercise of diabetic rats at this time point presumably as a consequence of general health decline due to the disease.

Therefore, voluntary running exercise in STZ rats prevents diabetic peripheral neuropathy progression, and it takes greater than 5 weeks for exercise to demonstrate this effect. Furthermore, these results could possibly indicate a delay in epidermal nerve fiber loss in small neurons, as STZ rats' progress from behavioural thermal hypoalgesia to fiber loss about 2 months later.

Table 2-4: Summary of viable animals

	SMALL			LARGE		
Healthy Sedentary	LCM cell #'s	RIN	[pg/ul]	LCM cell #'s	RIN	[pg/ul]
0965-1	1121	7.2	2319	732	6.8	1032
0965-2	1080	7.3	1342	869	6.8	1094
0966-1	1060	7.2	1605	835	7	892
0966-2	2200	7.1	537	1210	6.9	956
0977-2	1125	7.1	1035	849	7	1159
0978-2	1148	7.1	1183	820	7	2110
Diabetic Sedentary						
0968-2	1106	7.5	936	804	6.9	1578
0971-2	1645	7.6	871	822	6.9	1105
0974-2	1160	7.1	1298	813	6.5	1651
0975-2	1093	7.5	637	831	7.1	1038
0976-1	1655	7.7	798	880	NA	733
0976-2	1105	7.2	1977	843	6.9	1036
0979-2	1130	7.5	720	737	6.9	936
0980-2	1395	7.4	939	834	7	1123
0981-2	1115	7.2	1206	846	6.5	642
0982-2	1195	7.3	799	842	6.7	807
Diabetic Voluntary Wheel						
0963-2	1140	7.1	572	808	6.6	1765
0967-1	1155	7.3	1216	837	6.8	1713
0968-1	1145	7.3	1364	828	6.9	1613
0969-1	1285	6.8	917	808	6.5	873
0970-1	1235	7.5	1490	786	6.8	1334
0971-1	1128	7.6	943	810	7	2509
0973-1	1165	7.5	715	834	6.4	2121
0974-1	1130	7.3	923	850	7	1381
0979-1	1170	7.2	1835	810	6.4	2224
0980-1	1180	7.5	1177	806	7	1254
0981-1	1125	7.2	775	808	7.2	1239
0982-1	1170	7.3	971	780	6.8	1222

Legend:
LCM – Laser Capture Microdissection
RIN – RNA integrity number (please refer to general methods for further information)
"Viable" refers to the rats with enough LCM cells captured to allow RNA isolation, and those with good quality and concentration of RNA for further processing.

As indicated by the table above, out of the 12 rats that were diabetic and placed in a running wheel cage, all 12 were acceptable for analysis. In contrast, only 10 of the 12 animals that were diabetic and placed in a standard cage, and only 6 of the 12 animals that were healthy and placed in a standard cage were viable for analysis (i.e. sedentary rats).

All RNA samples were standardized at 537 pg/ul.

Figure 2-2: Voluntary wheel individual average running distance per week in STZ rat (14-15 weeks)
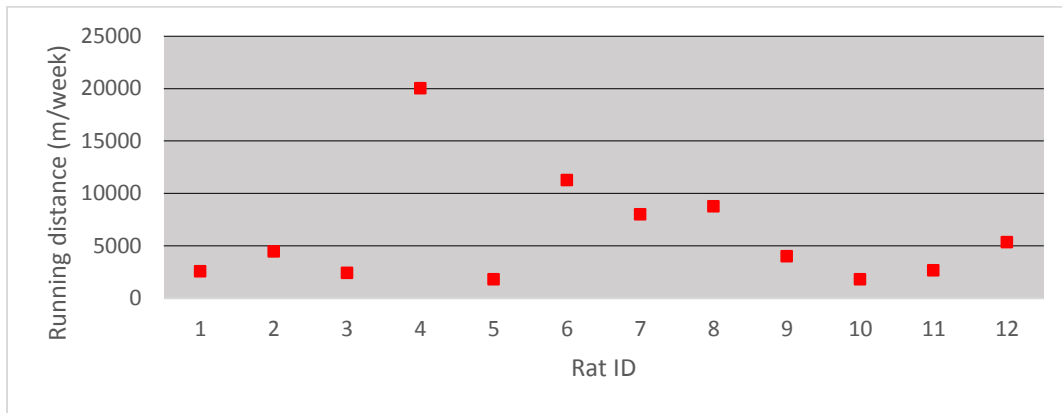
The STZ rat running the greatest distance, on average per week, was rat 4 at 20,017 m/week, and the lowest was rat 10 at 1785 m/week. Rat 6 had the highest running distance in one week at 38,093 m/week. Rats 1-3 and 5-7 and 10-12 decreased their average running distance per week from week 1 to their final week of running, while rats 4, 8 and 9 increased their running distance (for data please see appendix 5).

Figure 2-3: Voluntary wheel individual average running distance per week in healthy rat (15 weeks)
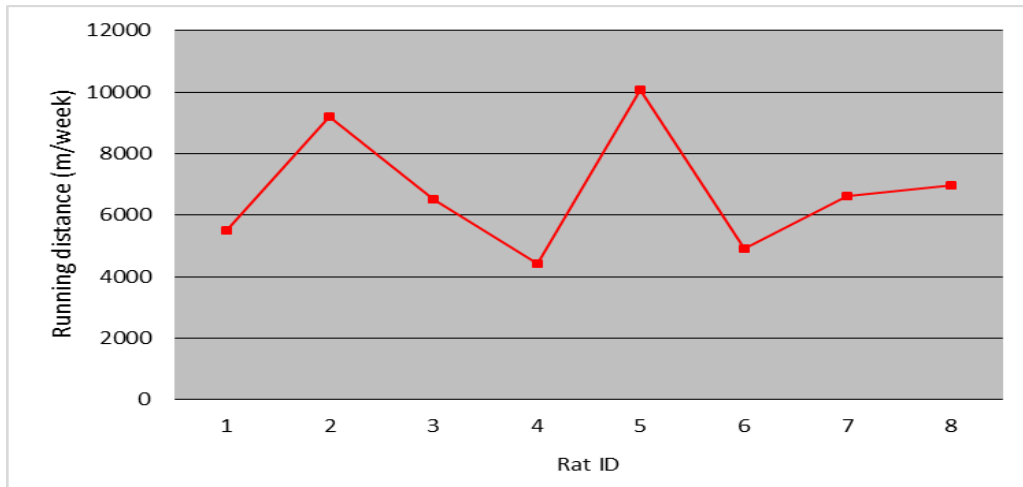
Compared to healthy rats with access to a voluntary wheel for 15 weeks, the average running distance per rat was 6774 m/week. Rat 5 ran the furthest distance per week on average at 10,077m. Rat 4 ran the least distance per week on average at 4403m.

Figure 2-4: Voluntary wheel group (n=12) average running distance per week in STZ rats (14-15 weeks)
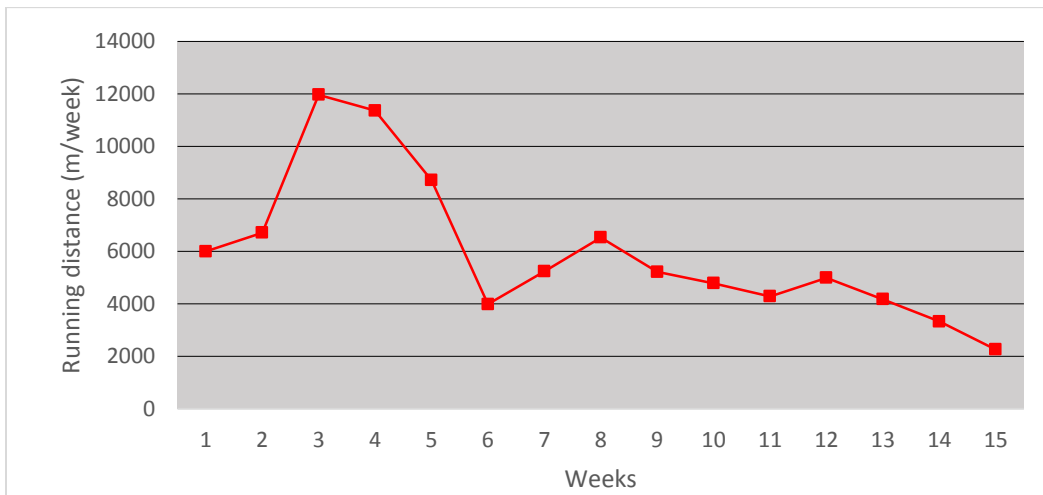
The STZ group's average weekly running distance was highest for week 3 at 11,968 m/week. The lowest average running distance for the group was week 14 at 3326 m/week. As you can see, on average, the group had a sharp increase in weekly running distance from week 1 to 3, and then gradually decreased to week 15 (with less running distance in weeks 14 and 15 compared to week 1).

Figure 2-5: Voluntary wheel group average running distance per week in healthy rats (15 weeks)
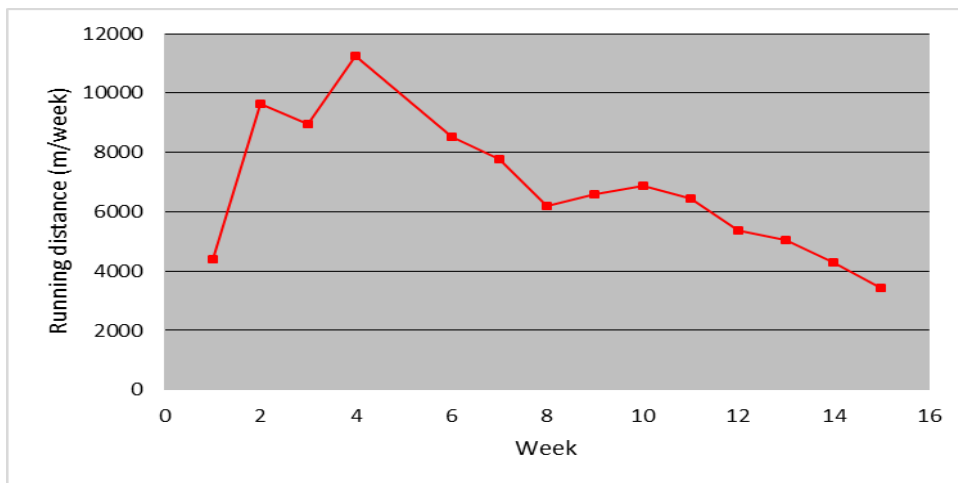
The groups average weekly running distance in STZ rats were similar to that in healthy rats (5969m vs. 6774m). The average running distance per rat per week was also similar (6075m vs. 6774m). The highest and lowest distance ran in one week on average was by a diabetic rat.

Therefore, even though diabetic rats as a group ran less weekly and total distances on average than healthy rats, their weekly and total individual distances showed greater variations and extremes.

Figure 2-6: Voluntary wheel total running distances in STZ rat (14-15 weeks)
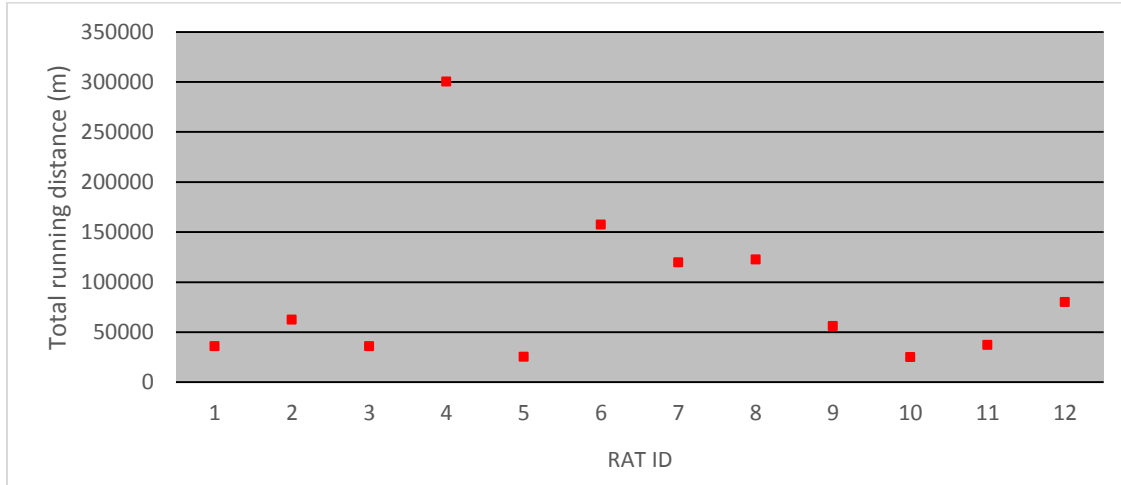
The average of the total distance ran by each STZ rat in 14-15 weeks (i.e. some rats were sacrificed during week 15) on a voluntary wheel was 88,025m. The sum of the average distances ran by the group per week in 14-15 weeks was 89,530m. The highest distance runner over the training period was rat 4 at 300,247m. The lowest distance runner over the training period was rat 10 at 24,990m (for data please see appendix 5).

Figure 2-7: Voluntary wheel total running distance in healthy rat (15 weeks)
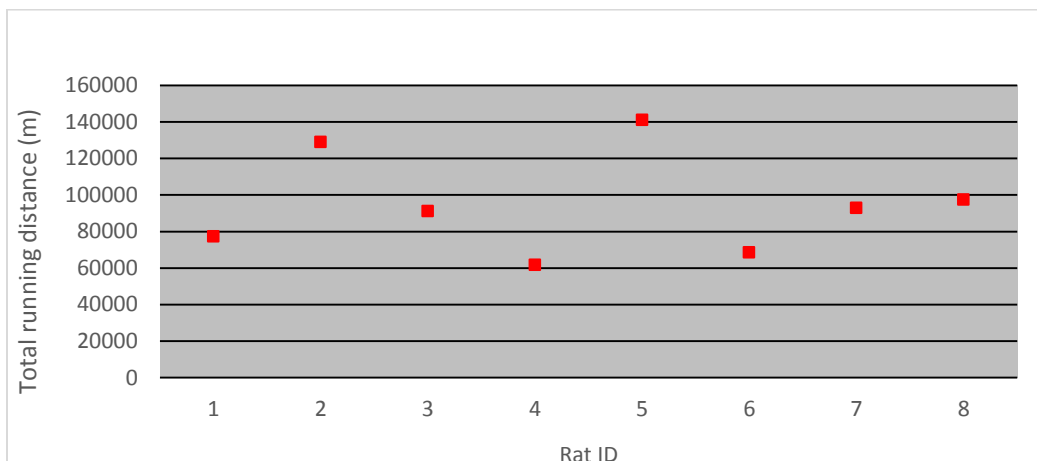
Compared to healthy rats with access to a voluntary running wheel, the average of the total distance ran by each rat in 15 weeks (i.e. they ran for 16 weeks in total) on a voluntary

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wheel was 94,832m. The sum of the average distances ran by the group per week in 15 weeks was the same. The highest distance runner over the training period was rat 5 at 141,079m. The lowest distance runner over the training period was rat 4 at 61,647m (for data please see appendix 5).

Therefore, diabetic rats ran 6807m less distance in total compared to healthy rats, considering the average of the sums of each rat's total distance. Diabetic rats ran 5302m less distance in total than healthy rats, when considering the sum of the average distances ran by the group per week. The rat with the longest total running distance was diabetic, and the rat with the lowest total running distance over the training protocol was also diabetic.

In healthy rats that exercised for 15 weeks on a voluntary wheel, the average running distance for the group was 6774 m/week. The group ran the most on week 4 at 11,252 m/week and the least on week 15 at 3449 m/week.

In summary, we have to take into account that some animals in study 2 only ran 14 weeks while some ran 15 weeks (i.e. due to ill health they were sacrificed early). Diabetes seems to have a negative effect on running distance for some rats, while not others. In general, diabetics ran less in total compared to healthy rats on average.

The effect of exercise on gene expression in diabetic rats

Figure 2-8: The effect of chronic voluntary wheel exercise on small DRG cell gene expression in STZ rats
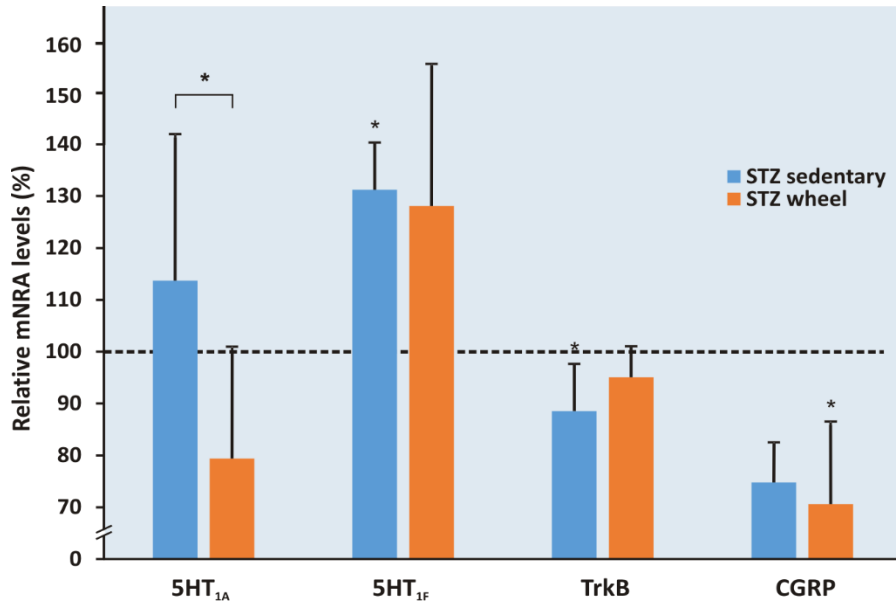
Legend:

Diabetic sedentary rats and Diabetic rats with 14-15 weeks of voluntary wheel exercise show differences in mRNA levels in small DRG cells. mRNA levels in the small L4–5 DRG cells were measured by real-time RT-PCR. All values were normalized for the amplification of SDHA. Standard deviation bars = SD for 6-12 animals per condition. mRNA levels with STZ sedentary and STZ wheel are expressed as a percentage relative to the healthy sedentary animals (healthy sedentary represented by ------ at 100%). Statistically significant differences between healthy sedentary and STZ sedentary or STZ exercised animals are indicated by an * above the bar. Line over bars with an * on top indicate statistically significant differences between STZ sedentary and STZ exercised animals. p < 0.05 (*) by ANOVA and Tukey post-hoc test.

There were no statistically significant differences in large DRG cell gene expression in STZ sedentary or STZ exercised rats (see appendix 6 for complete data).

In small cells (Figure 2-8; see appendix 6 for complete data) of STZ sedentary rats, 5HT1F mRNA levels were higher, and TrkB was lower, compared to healthy sedentary rats. In small cells (Figure 2-8) of STZ exercised rats, CGRP was down-regulated compared to healthy sedentary rats. 5HT1A expression was not significantly different from healthy sedentary rat mRNA levels, however, the receptor was more highly expressed in STZ sedentary compared to STZ exercised rats (Figure 2-8).

The results indicate that 14-15 weeks of voluntary wheel exercise was effective in lowering the level of 5HT1F transcript and preventing the decrease in TrkB receptor levels associated with diabetes. Furthermore, diabetic rats that exercised showed a down-regulation in 5HT1A relative to diabetic sedentary rats, and a decrease in CGRP mRNAs that was not evident in STZ or healthy sedentary rats.

The gene expression regulatory effects of both STZ and STZ plus exercise at 14-15 weeks (Figure 2-8), may be related to the behavioural testing results noted at 10-11 weeks (Figure 2-1). Low intensity exercise of 8-11 weeks, is positively correlated with beneficial effects on DPN.

DISCUSSION

The STZ rat model was used to examine the effects of exercise on diabetes. Our hypothesis was that diabetic rats that exercise would experience a delay in the onset of thermal hypoalgesia, as well as corresponding changes in DRG gene expression that might provide a suggestion for a molecular mechanism(s) to support this result. It is apparent that exercise caused a partial prevention/reversal in thermal hypoalgesia in this study, since diabetic wheel-trained rats did not significantly differ in withdrawal latency compared to healthy sedentary animals at 8-11 weeks of diabetes, whereas diabetic sedentary rats did. Multiple genes were regulated in small DRG neurons, which offer insight into potential supporting mechanisms.

Diabetic peripheral neuropathy, and how it is affected by exercise, is reviewed in the introduction (Please see pages 35-40).

Changes in gene expression at the DRG due to diabetes and exercise

Electrical activity and molecular signalling in the DRG activate second messenger cascades that result in transcription of molecules that are then transported to both the central and peripheral terminals where they are released out of the terminal or inserted into the membrane to regulate synaptic strength and excitability (Woolf & Costigan, 1999). The response of transcriptional changes at the DRG is caused by an increase in activity at the DRG as well as retrograde transport of signal molecules from the periphery to the DRG, such as TrkB/BDNF (Mantyh et al, 2011). The increased synthesis of neuropeptides such as CGRP, and receptors such as TrkB, 5HT1A and 5HT1F, leads to the anterograde transport of these molecules from the

DRG to the spinal cord and peripheral terminal membranes (Mantyh et al, 2011; Woolf & Costigan, 1999).

It is likely that changes in mRNA levels are reliable indicators of changes in gene expression in our studies (Please see intro pgs. for more detail). It is understood however, that in all cases mRNA levels may not reflect protein levels, and hence the functional significance in the sensory system cannot be definitively determined. Further work would have to be completed to see if protein mirrors mRNA in these models. However, a further consideration of measuring protein instead of mRNA in DRG cells is that a protein of interest is not necessarily located in the soma and can be located in other areas of the neuron. A protein study could include semi-quantitative immunofluorescence where density of a particular protein is measured.

Voluntary wheel exercise results in lower CGRP mRNA levels, in small DRG cells, in diabetic compared to healthy sedentary rats. CGRP is a member of the calcitonin peptide family. It is produced in both central and peripheral neurons, including DRG (Rosenfeld et al, 1983). Centrally, CGRP is found in both dorsal and ventral horns of the spinal cord (Chen et al, 2010). In C- and Aδ-axon terminals of the dorsal horn, CGRP participates in pain transmission via activation of CRL and RAMP1 receptors (Poyner et al, 2002). CGRP is regulated in various ways including increased calcium influx leading to a decrease in CGRP promoter activity (Durham et al, 2003) and TRPV1 activation which causes CGRP release (Meng et al, 2009). In addition, NGF is necessary for increases in CGRP in both the DRG and nerve terminals (Diemel et al, 1994; Sango et al, 1994). Once CGRP is synthesized, it is stored in large dense core vesicles within sensory nerve terminals. Neuronal depolarization causes release from terminals via calcium depolarized-exocytosis (Russell et al, 2014). For example, a skin stimulus can result

in dorsal horn CGRP release leading to central sensitization and increased pain and/or an axon reflex causing CGRP release in the arteriole leading to vasodilation and flare (ie. neurogenic inflammation) (Russell et al, 2014).

Three weeks of treadmill training was previously found to increase CGRP expression in healthy whole DRG, whereas one week of training showed no change (Sun & Pan, 2014). Study 1 results did not show any changes in CGRP mRNA levels in small or large cells after acute or chronic treadmill, or after chronic voluntary exercise in healthy rats. Therefore, our results agree with Sun & Pan for the 1 week exercise study, but inconsistent with the 3 week study. We did not examine gene expression at 3 weeks of exercise (i.e. only 1 week and 16 weeks), so we can't compare. But our results suggest that the increase in CGRP reported by Sun & Pan may be most evident in medium DRG cells. Furthermore, DRG neurons respond differently to exercise in a diabetic verses a healthy rat model. Endurance exercise was also found to increase CGRP levels in motoneurons, sciatic nerve (Gharakhanlou et al, 1999; Homonko & Theriault, 1997), plasma (Onuoha et al, 1998; Schifter et al, 1995), muscle (Parnow et al, 2012; Schuler et al, 2014) and CSF (Wyon et al, 1998) in healthy rats.

In contrast, increases in CGRP caused by an abnormal state, such as migraine, results in pain. Exercise is a prescribed treatment for certain types of migraine, by causing a decrease in CGRP release in trigeminal neurons (Hindiyeh et al, 2013). We also know that 5HT1 agonists such a sumatriptan used to treat migraine pain, also cause a decrease in CGRP promoter activity (Durham & Russo, 2003), and that exercise increases the inhibitory actions of 5HT1 receptors. CGRP and its receptor antagonists are also used to effectively alleviate migraine (Russell et al, 2014).

Limited research has been conducted with regards to the effect of endurance exercise on CGRP levels in diabetes, but circulating levels were shown to both increase (Lind et al, 1996) and not change in plasma after chronic treadmill training (Daneshyar et al, 2014) in diabetics. Interestingly, TENS used on diabetics results in an increase in CGRP mRNA and protein in DRG (Ding et al, 2013). However, it is unclear how exercise effects CGRP levels in the DRG.

Diabetes resulted in reduced CGRP levels in the heart (Zheng et al, 2012), pancreatic islet cells (Al-Salam et al, 2009), and serum (Wang et al, 2012; Yang et al, 2013). The effects of diabetes on CGRP levels in the PNS, seem to be time-dependent. The timeline of DPN progression is relevant to both gene expression and behavioural results. At 14-28 days of diabetes in STZ rats, there was an increase in CGRP labelling in whole DRG and dorsal horn (Kou et al, 2014). This increase was associated with mechanical allodynia and impaired locomotor activity. However, 4-6 weeks of diabetes in STZ rats, resulted in decreased CGRP mRNA levels in whole DRG (Diemel et al, 1994; Tomlinson et al, 1997). In another study by Zochodne et al (2001), STZ rat whole DRG did not show any change in CGRP mRNA levels at 2 months of diabetes, but at 12 months of diabetes levels were decreased. Therefore, it seems during the early stages of experimental diabetes, CGRP levels in the DRG are decreased. However, as the disease progresses, CGRP levels switch from an increase to a decrease in the DRG, which may correspond to a switch from hyperalgesia to hypoalgesia in STZ rats.

One theory for painful diabetic neuropathy is an increase in CGRP in TrkA positive intra-epidermal nerve fibers (Evans et al, 2012) as well as abnormal stimulation to sensory nerves and constant C-fiber discharge causing spinal sensitization (Fuchs et al, 2010). On the other hand, with the loss of nociceptive sensation, which results in lack of wound healing and ulcers, there is a decrease in NGF leading to less CGRP containing sensory neurons (Russell et al, 2014).

Furthermore, there is also small fiber loss in the skin associated with the nerve endings dying back, with peptidergic fibers going first (Johnson et al, 2008). This cutaneous fiber loss is the main cause of insensate diabetic neuropathy.

Our results show that CGRP mRNA levels were not significantly different in DRG cells of diabetic sedentary compared to healthy sedentary rats at 14-15 weeks of diabetes. Though not statistically significant, CGRP mRNA levels in diabetic sedentary animals were down-regulated by 25% compared to healthy sedentary controls. This result is not consistent with previous findings of the effect of diabetes on CGRP levels in the DRG. However, our results are the first to use the novel method of single cell laser capture microdissection to examine small cell mRNA specifically. Therefore, it is possible that whole DRG levels of CGRP would be decreased, but this attenuation may not be evident in the small or large cell population. This would suggest that CGRP levels are mainly decreased in the medium sized DRG cells with diabetes. Hoffman et al (2011) used a NGF antagonist resulting in hypoalgesia via a decrease in CGRP in medium DRG neurons. In addition, DRG may have other sources of CGRP that are not neuronal, which may be affected by DPN and could contribute to changes in CGRP levels in whole DRG samples. It is also possible that CGRP levels fluctuate throughout diabetes progression. Our study seems to be the first to examine CGRP levels in the DRG of STZ rats at $3.5 - 4$ months of diabetes. Previously, CGRP levels were found to be increased at 2 weeks-1month of diabetes (Kou et al, 2014), unchanged at 2 months (Zochodne et al, 2001), and decreased at 1-1.5 (Diemel et al, 1994; Tomlinson et al, 1997) and 12 months (Zochodne et al, 2001) in whole DRG samples.

Diabetic rats that exercised on a voluntary wheel for 14-15 weeks displayed significantly lower CGRP mRNA levels (29% less), in small DRG cells, compared to healthy sedentary rats. Therefore, 14-15 weeks of diabetes was not sufficient to induce a significant change in CGRP

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mRNA levels in small cells, whereas chronic voluntary wheel exercise apparently enhanced the response of small DRG cells to diabetes. Since lower CGRP levels are associated with anti-nociception in pain models (Hindiyeh et al, 2013; Russell et al, 2014), and loss of nociceptive sensation in DPN (Russell et al, 2014), the down-regulation of CGRP with exercise is conducive to hypoalgesia (ie. anti-nociception/decrease in pain transmission). However, this is conflicting to the positive behavioural results at 10-11 weeks of diabetes (i.e. prevent/reverse thermal hypoalgesia). It is possible that at that time point, CGRP levels were not changed by exercise, but 3-5 weeks later when animals were sacrificed and gene expression studies were carried out, levels were changed. Hence, the gene expression result may not be representative of the prevention of thermal hypoalgesia induced by exercise.

Since we do not have a whole DRG model to compare to with regards to effect of exercise in chronic diabetes, it is difficult to ascertain what a decrease in CGRP in small cells with chronic voluntary exercise could mean. Since medium DRG cells may be the main contributor to the decrease in CGRP levels reported in other diabetic studies, and since our study did not show any significant change in CGRP levels in small or large cells with diabetes, our result of a decrease in CGRP levels in diabetics with exercise, may mean that diabetic small cells are more affected by exercise than non-exercised. Likewise, it is possible that exercise causes a switch in neuronal phenotype, so that there is less CGRP positive small neurons and more CGRP positive medium DRG neurons in diabetics (i.e. similar to what happens with BDNF in Cao et al, 2010). Furthermore, the initial hyperalgesia response, and /or the early peptidergic fibre loss in DPN could cause or be related to these changes with exercise. Exercise causes a decrease in CGRP, similar to causing decrease associated with decrease in pain in other studies.

Voluntary wheel exercise seems to have a different effect on CGRP levels in small and large DRG cells in healthy compared to diabetic states. It is not clear what this change means functionally or in relation to the behavioural results, but probably indicates cutaneous insensitivity (Johnson et al, 2008). Hence, the lack of pain sensation due to diabetic neuropathy may, at least in part, be caused by a decrease in CGRP signalling. However why this is not reported in the STZ sedentary rats who demonstrated thermal hypoaglesia, but was evident in the exercised rats who did not demonstrate thermal hypoalgesia at 8-11 weeks of diabetes, is not clear and requires further investigation.

Voluntary wheel exercise prevents the diabetic-induced attenuation of TrkB mRNA in small DRG cells. TrkB receptor mRNA levels were significantly lower in small DRG cells of diabetic sedentary compared to healthy sedentary rats. However, diabetic rats that exercised on a voluntary wheel for 14-15 weeks displayed no difference in TrkB receptor gene expression, in small DRG cells, compared to healthy sedentary rats. Therefore, apparently, chronic voluntary wheel exercise prevented the decrease in TrkB receptor mRNA in small DRG cells caused by diabetes. This change in DRG gene expression seems to correspond to positive behavioural results at 10-11 weeks diabetes. At this time-point, the average withdrawal latency was significantly longer in diabetic sedentary rats compared to healthy sedentary rats, whereas diabetes rats that exercised displayed no difference in latency compared to healthy sedentary rats. These results indicate that voluntary wheel exercise prevented/reversed thermal hypoalgesia in STZ rats.

There is limited previous research concerning the effect of diabetes on TrkB receptor levels in DRG neurons, and none involving exercise. In sciatic nerve of 6 week diabetic rats,

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TrkB receptor mRNA levels were decreased by 50%, but returned to control levels after 12 weeks (Rodriguez-Pena et al, 1995). In another study by Zochodne et al (2001), lumbar DRG neurons from STZ rats exhibited no change in TrkB mRNA levels at both 2 and 12 months. However, there was a decrease in motor and sensory nerve conduction velocities at both time-points, whereas axonal atrophy and changes in expression of other genes at the DRG were not evident till 12 months. There was no change in DRG cell numbers. These conflicting results between studies may be attributed to the variations in time point for gene expression analysis (i.e. 6 and 12 weeks vs. 2 and 12 months), as well as the examination location on the neuron (i.e. nerve vs. cell body). In comparison to our results, it is possible that diabetes causes a change in DRG gene expression at 14-15 weeks that is not evident at 2 months, but not in peripheral nerve. It is also possible that no change in TrkB receptor mRNA levels would be observed in whole DRG samples, yet expression levels changed in small cells. TrkB receptors are present on small, medium and large DRG cells (McMahon et al, 1994). Therefore, even though we did not find any change in TrkB receptor mNRA levels in large cells, it is possible that a change in mRNA levels in small cells could be "masked" by change in medium cells (i.e. excluded in this study), that would result as no change in a whole DRG sample. It is also possible that whole DRG samples contain non-neuronal sources of TrkB which would skew the results. Another consideration is that the decrease in TrkB receptor expression may not be evident till 14-15 weeks, and the change may disappear by 12 months.

Target tissues, such as skin and muscle, release BDNF, which then binds to the TrkB receptor on the peripheral nerve terminal. It was previously reported that 50% of muscle afferents express TrkB in mainly large fibers (i.e. some small too), and 25% of cutaneous afferents are TrkB positive in both small and large fibers (McMahon et al, 1994). The

receptor/ligand complex is then internalized and retrogradely transported to the DRG cell body (Apfel, 1999). BDNF is also synthesized in the DRG cell body and anterogradely transported both centrally and peripherally. At the soma, TrkB is auto-phosphorylated and signalling occurs via 2nd messenger transduction pathways (i.e. MAPK, PI3K), which regulate transcription. TrkB receptors are also present on and signal at neurons that are post-synaptic to C-fiber afferents in the dorsal horn, and are present at primary afferent terminals, but whether they signal, and their role, is unclear. (Pezet et al, 2002). Hence, BDNF release from target tissues or primary afferent terminals can activate TrkB receptors. Furthermore, Tonoli et al (2015) found that humans with type 1 diabetes exhibit an increase in serum BDNF levels. Since systemic circulation can access DRG neurons, it is also possible that BDNF binds directly to and activates TrkB receptors on the cell body (Apfel, 1999).

TrkB specifically binds BDNF and NT4 (Ip et al, 1993). Therefore, changes in these neurotrophins with diabetes can provide insight into possible implications associated with the TrkB receptor changes. In STZ rat at 12 weeks, BDNF mRNAs are increased in soleus muscle and lumbar DRG (Fernyhough et al, 1995). The up-regulation of BDNF was suggested as a protective mechanism induced by early diabetic changes/stresses. Mizisin et al (1999) used sciatic nerve ligatures in STZ rats to show that there were no changes in anterograde or retrograde BDNF accumulations. They also found that BDNF injection to the sciatic nerve did not result in any change to retrograde transport to the DRG. Another study found lower BDNF levels, but no change in NT4 mRNAs, in the skin of humans with diabetic neuropathy compared to controls (Sinicropi & Lazarus, 2001). These results are not substantiated in STZ rats to my knowledge. However, in normal mice, BDNF protein was present in sciatic nerve, spinal cord, DRG, gastrocnemius muscle and hind-paw skin (Groover et al, 2013). In mice fed a high fat diet

to induce pre-diabetes, BDNF levels did not significantly change. However, in both normal controls and pre-diabetic mice, 12 weeks of voluntary wheel exercise increased BDNF protein in the sciatic nerve only, although the pre-diabetic mice showed a significantly lesser increase in BDNF compared to controls. Furthermore, there was a decrease in NT4 mRNA levels in rat sciatic nerve after 12 weeks of diabetes (Rodriguez-Pena et al, 1995). Our study showed BDNF gene expression levels in small and large DRG cells were not significantly altered with diabetes or exercise. However, a limitation was that we did not include medium size cells, which normally contain BDNF (Zhou & Rush, 1996; Thompson et al, 1999). Therefore, just because we did not see an increase in small or large cells does not mean that BDNF does not increase in the DRG, and suggests that the increase found by Fernyhough et al, may have been primarily in medium cells. BDNF is present in some small and medium cells of healthy animals (Zhou & Rush, 1996, Thompson et al, 1999), but diabetes caused an increase in number of DRG cells that express BDNF, and a shift to most medium and large cells containing BDNF (Cao et al, 2010). Hence, it is possible that less BDNF in small cells with diabetes could also cause a decreased need for TrkB receptors in those cells. Cao et al (2010) also found that the increase in BDNF and its signalling via TkrB receptors in medium-large neurons, led to decreased Kv expression and currents related to increased excitability of DRG neurons, and pain by 3 weeks. Therefore, the opposite could be true with a decrease in TrkB signalling resulting in less excitability of these cells, reducing/removing the pain response. This reasoning corresponds with our behavioural result of thermal hypoalgesia in STZ sedentary rats. It also supports the prevention of the diabetes-induced decrease in TrkB receptors by exercise correlating with the prevention/reversal of thermal hypoalgesia caused by exercise.

Similar to TrkA/NGF signalling, it may be possible that a decrease in the TrkB receptor in small cells would lead to decreased retrograde axonal transport of BDNF/NT4, and hence decreased support of BDNF-dependent sensory neurons, and decreased gene expression. (Tomlinson DR et al, 1997). A decrease in TrkB receptors in small DRG neurons of STZ rats could lead to decreased BDNF signalling in the soma, with a resultant decrease of available BDNF to be transported to the central or peripheral terminals (Zhou et al,1993; Michael et al, 1997). Our study showed 28% less BDNF mRNA in STZ sedentary, and only 13% less in STZ voluntary wheel, compared with healthy sedentary rat small DRG cells, however, these differences were not statistically significant. Centrally, release of BDNF from stimulated C-fiber terminals (Lever et al, 2001) can activate TrkB receptors on post-synaptic neurons in the dorsal horn causing post-synaptic excitability and nociceptive transmission (Pezet et al, 2002). Furthermore, BDNF antagonism decreases thermal hyperalgesia associated with inflammatory pain (Pezet et al, 2002). Therefore, a decrease in BDNF release could mean less TrkB receptor activation post-synaptically, and an attenuated pain response, which is consistent with our behavioural data. Hence, the prevention of diabetes-induced attenuation of TrkB receptor mRNA with voluntary wheel exercise, could mean normal BDNF signalling at both the DRG cell body and dorsal horn neurons (i.e. pre-synaptic signalling unclear), supporting the prevention/reversal of thermal hypoalgesia induced by diabetes.

Compared to NGF in particular, and even NT3, relatively little is known regarding BDNF/TrkB related to the pathogenesis of DPN. STZ rats display both MNCV and SNCV slowing by 1-2 months (Arezzo & Zotova, 2002), and small fiber loss in the skin by 6 months (Johnson et al, 2008), representing diabetic neuropathy. Since our study showed thermal hypoalgesia at 5-11 weeks in STZ sedentary rats, and a decrease in TrkB receptor gene

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expression in small DRG cells at 14-15 weeks, it may be too early to suspect it representing small fiber loss, but could be a result of the pathogenesis related to the slowing of the SNCV and the thermal hypoalgesia. However, 14-15 weeks of voluntary wheel exercise prevented/reversed these changes as early as 8 weeks of exercise, and hence, TrkB receptor levels were maintained up to 14-15 weeks.

5HT1A receptor mRNAs are more highly expressed in small DRG cells of diabetic sedentary compared to diabetic exercised rats. 5HT1A receptor mRNA levels in diabetic sedentary or diabetic exercised rats were not significantly different from healthy sedentary controls. However, 5HT1A receptor gene expression was significantly higher in small cells of diabetic sedentary compared to diabetic exercised animals. These results seem to correspond with our behavioural testing results. This is the first results of its kind with regards to how 5HT receptors in small and large cells of the DRG are affected by diabetes and exercise.

There is a relative dearth of research examining 5HT1A receptors and diabetes, with the majority of work conducted in the brain. For an in-depth review of 5HT and 5HT1A in healthy sedentary and exercised animals please refer to study 1 discussion. Basically, 5HT1A receptors play an inhibitory role in Aδ and C nociceptive fibers both peripherally and spinally in both intact and neuropathic animals to decrease pain. Low expression of the 5HT-transporter in humans, is related to a higher threshold to thermal pain and hypoalgesia (Lindstedt et al., 2011). Furthermore, exercise has been shown to increase 5HT levels in the brain. We suggest these exercise-induced changes contribute to analgesia via more 5HT being released from the descending pathway onto more 5HT receptors in the dorsal horn that act to inhibit pain transmission (i.e. 5HT1D and 1A receptors).

In diabetes, the changes related to serotonin and the 1A receptor depend on the location. With chronic STZ-induced diabetes, there seems to be dysfunction in brain monoamine transmission (Petrisic et al, 1997). There is also an increase in 5HT1A receptors in brainstem and cerebral cortex (Price et al, 2002; Sandrini et al, 1997), and a decrease in 5HT in hypothalamus, brainstem (Chu et al, 1986), and cerebral cortex (Price et al, 2002; Sandrini et al, 1997). Furthermore, Morgado et al (2011) showed an increase in 5HT in the dorsal horn of the spinal cord, and an increase in tryptophan hydroxylase at the rostral ventral medulla, which may lead to enhanced pain. Others showed a decrease in 5HT turnover in the brainstem (Henley & Bellush, 1992) and 5HT release at the spinal cord. However, no change was evident in 5HT content in the lumbar spinal cord (Suh et al, 1996). Furthermore, diabetics showed no effect on 1A receptor density in the hippocampus (Sumiyoshi et al, 1997). 5HT synthesis has been shown to decrease (Price et al, 2002) in hypothalamus (King & Rohrbach, 1990), while its transporter shows an increase in dorsal raphe (Price et al, 2002; Petrisic et al, 1997) with diabetes.

The decrease in 5HT concentration in the brain may be compensated by an increased number of 1A receptors (Sandrini et al, 1997; Price et al, 2002). The behavioural abnormalities sometimes associated with diabetes such as decreased motor activity, mood alterations, sex drive changes and anxiety are thought to be contributed by these changes in 5HT transmission (Sandrini et al, 1997). Mechanical hyperalgesia and chemical allodynia associated with painful diabetic peripheral neuropathy are also thought to be caused by an increase in 5HT resulting in neuronal hyperactivity at both the spinal cord dorsal horn and periaqueductal gray (Morgado et al, 2011). It is interesting to note that diabetes shows preservation of long 5HT axons innervating spinal cord and cerebral cortex with even hyperinnervation to the hypothalamus, but there is attenuation of shorter collaterals in the pons and medulla oblongata (DiGiulio et al, 1989).

A study by Wirshing et al (1998) proposed that using an 1A receptor antagonist causes attenuated beta-cell responsiveness leading to low insulin and hyperglycemia, and subsequent new onset of diabetes. Alternatively, using a 1A receptor agonist on diabetics can have a beneficial effect on lumbosacral spinal cord transmission for example, with increased voiding efficiency and decreased external sphincter EMG activity (Gu et al, 2012). 5HT re-uptake inhibitors are successfully used to treat painful diabetic peripheral neuropathy (Tesfaye et al, 2011; Ikeda et al, 2009). 5HT1 receptors (the particular subtype was not investigated), when activated centrally (did not distinguish pre- and post-synaptic), were shown to have an anti-nociceptive effect on painful diabetic neuropathy in mice (Takeshita & Yamaguchi, 1995).

However, the STZ rat model we used produces thermal hypoalgesia at 5-11 weeks of diabetes. The 5HT1A receptor can also have hypoalgesia effects (Matsuda, 2013). Chu et al (1986) showed an increased pain threshold in STZ animals. It is not surprising that diabetic sedentary animals would show an increase in 1A receptor expression at the DRG, as it does also in the spinal cord and brain. The potential functional implication for the result is associated with an inhibitory action or less pain transmission in healthy animals, and corresponds to thermal hypoaglesia. Hence, in diabetics, activation of the 1A receptor has been shown to result in painful and insensate peripheral neuropathy. Therefore, the function of 5HT1A receptor activation in diabetes may be time dependent (i.e. DPN progression). Early on when hyperalgesia/allodynia is present, the 5HT1A receptor may act to elicit pain, may be due to a compensatory mechanism for the decrease in pre-synaptic 5HT concentration and synthesis. However, later when hypoalgesia is present, the 1A receptor may actually increase the pain threshold. Therefore, greater 5HT1A receptor expression in small cells of STZ sedentary compared to STZ voluntary wheel rats is consistent with the thermal hypoalgesia response.

Likewise, less 5HT1A receptor mRNAs in small cells of STZ exercised compared to STZ sedentary rats, may contribute to the exercise-induced prevention/reversal of thermal hypoalgesia.

Voluntary wheel exercise lowers the diabetic-induced up-regulation of 5HT1F receptor mRNA in small DRG cells. 5HT1F receptor mRNA levels in small cells of diabetic sedentary rats were significantly higher relative to healthy sedentary controls. However, 5HT1F receptor gene expression was not significantly different in small cells of diabetic exercised compared to healthy sedentary animals. Hence, it appears that exercise prevented the diabetes-induced increase in 5HT1F receptor mRNAs, in small DRG cells. This change in DRG gene expression corresponds positively to our behavioural results at 8-11 weeks diabetes. These results indicate that voluntary wheel exercise prevented/reversed thermal hypoalgesia in STZ rats, and the prevention of the diabetes-induced 5HT1F receptor increase by exercise supports this finding.

To our knowledge, there is no prior information regarding 5HT1F receptors involvement in exercise response. There is also very limited research available related to 5HT1F receptors in general, compared to other 5HT receptors and their actions in the nervous system. Most studies have focused on 5HT1F receptors anti-migraine effects. For example, Classey 2010 and Agosti 2007 show that 5HT1F receptors acting at the TGN have an anti-migraine effect, but they also suggest that due to the receptor's presence in DRG cells, that they could be targeted for treatment of other types of pain as well. Furthermore, the use of a 5HT1F agonist produced antinociception in the rat formalin test, causing a reduction in inflammatory pain (Granados-Soto et al, 2010). Hence, in a pain model, activation of the peripheral 5HT1F receptor results in anti-nociception.

Our results show that regulation of the 5HT1F receptor is decreased in small cells after chronic treadmill exercise, indicating its potential role in altering nociceptive transmission.

Centrally, 5HT1F receptors seem to be confined to primary afferent terminals, and play an anti-nociceptive role in the spinal cord dorsal horn (Millan, 2002; Castro et al, 1997). Similar to other 5HT1 receptors, 1F acts to inhibit pain transmission via activation of Gi coupled proteins that inhibit adenylate cyclase, thereby inhibiting intracellular cAMP (Boess & Martin 1994; Millan, 2002). The majority of the 5HT response in primary afferent terminals is in C-fibers (Hagashi et al, 1982; Stansfeld et al, 1982; Fock & Mense, 1976), where the activated 5HT receptor, appears to function by decreasing glutamate release (i.e. PSI), causing anti-nociception (Yoshimura & Furue, 2006). Hence, activation of the pre-synaptic 5HT receptor, influences post-synaptic neuronal firing, and thus, spinal transmission of sensory messages. In large muscle afferents, the 5HT1F receptor was suggested to act pre-synaptically to decrease EPSPs after SCI, resulting in reflex inhibition and spasm control (Murray et al, 2011). Other studies indicate that 5HT receptors may act pre-synaptically in large afferents, influencing neurotransmitter release and PSI (Perrier & Cotel, 2015; Dougherty et al, 2005; Gosgnach et al, 2000). However, the particular pre-synaptic metabotropic receptors have not been identified.

In summary, 5HT1F receptors are present on peripheral and central terminals, and at the DRG soma. In pain models, activation of the receptor results in anti-nociception at the periphery and centrally. An increase in the 5HT1F receptor in small cells, suggests that diabetes increased the anti-nociceptive response, which is consistent with our thermal hypoalgesia result. Chronic voluntary wheel exercise acts to prevent this increase, and thus, normalize nociceptive transmission in diabetic animals. Therefore, exercise may act to prevent thermal hypoalgesia

associated with DPN, and one mechanism supporting this response is the prevention of increased 5HT1F receptor.

Conclusions

Our study shows that voluntary wheel exercise has a beneficial effect on diabetic rats evidenced by a prevention/reversal in the progression of thermal hypoalgesia. We also found multiple differences in gene expression in STZ sedentary and STZ wheel study groups at the DRG, and more specifically, small DRG neurons. Hence, small DRG neurons are apparently more responsive or affected by DPN and exercise than large neurons. No general patterns of gene expression were evident. However, the changes in 5HT1A, 5HT1F and TrkB receptor mRNA seem to correlate well with the exercise-induced prevention/reversal of thermal hypoalgesia, while the role of the decrease in CGRP mRNA levels with exercise is less clear. These differences suggest potential mechanisms underlying changes in nerve function and loss related to DPN, and can help provide insight and direction on new and modulated treatments for diabetics.

GENERAL DISCUSSION

This thesis comprises two studies in which gene expression of small and large DRG neurons was examined. The first study involved responses to acute and chronic exercise in healthy rats, while the second study incorporated differences imposed by diabetes, and how those differences were affected by chronic exercise. Eight key highlights arose from these studies: 1) most of the genes examined differ in their expression between small and large DRG neurons; 2) this is the first study to show differences in gene expression in small and large DRG neurons with exercise; 3) DRG neurons respond differently to different forms of aerobic exercise (ie.voluntary vs forced); 4) acute and chronic exercise differ in their effects on small and large DRG neuron gene expression; 5) voluntary wheel exercise prevents/reverses the progression of thermal hypoalgesia in diabetic rats; 6) there are gene expression differences in small and large DRG neurons with diabetes; 7) exercise changes certain gene expression differences produced by diabetes. The gene expression differences elicited by exercise and diabetes have several possible implications including changes in neurotransmitter release and hence altered transmission of afferent information, neuron excitability, nerve conduction velocity, nerve regeneration, muscle strength, motor output and others. Specific small and large DRG cell gene expression changes were already discussed in detail in the respective studies; therefore, this general discussion will focus on broader findings, limitations, and future studies.

Large and small DRG neurons varied in their sensitivity to exercise. This is very important to our understanding of what these differences in gene expression mean from both a mechanistic and functional perspective. If there is a difference in gene expression for a particular gene in a large neuron for example, that gives an indication of what type of sensory transmission

is potentially affected. Therefore, the overall potential impact of exercise on DRG neurons is modification in nociceptive and proprioceptive signaling (i.e. synaptic efficacy and excitability). Furthermore, the majority of genes examined are normally more highly expressed in small DRG neurons. This likely means they are more functionally relevant to nociceptive processing compared to other genes more highly expressed in large neurons that would be more involved in other forms of afferent signaling (although there are exceptions).

Of the genes that were investigated in Study 2, there were no significant differences in expression in large DRG neurons induced by diabetes. However, 2 of the 15 genes tested did show expression differences in small DRG neurons in diabetic compared to healthy sedentary animals. In addition, 1 gene in diabetic rats that exercised was significantly different in expression than diabetic sedentary rats in small neurons. Similarly, voluntary wheel exercise by diabetic rats displayed no significant differences in expression in large DRG neurons, and only one gene in small neurons compared to healthy animals. These findings could mean small DRG neurons are more susceptible or sensitive to changes, than are large neurons, in response to diabetes. Lastly, it is possible that small neurons are more responsive to or affected by exercise in terms of causing significant change in diabetics. Therefore, results of differences in gene expression in small, and lack of change in large DRG neurons, and their meaning should be interpreted carefully before making broad and general statements concerning the effect of exercise on diabetes pathophysiology, as well as specific prescriptions regarding exercise treatment in diabetic humans.

There were limitations within each study presented. Firstly, the neuron size restrictions we determined for LCM may not be completely physiologically realistic (ie. 30um or less used to

classify small DRG neurons could potentially include some Aβ fibers; 40um or greater used to classify large DRG neurons could potentially include some Aδ fibers). Hence, discussion of different types of signals relayed from the periphery could be inaccurate. However, the classifications we used in our studies were consistent with the literature including work done by others as well as the establishment of small and large classification according to conduction velocity differences (ie. exact "cut-off" diameters between each fiber type does not seem to be as well established). Secondly, running distance differences between healthy and diabetic animals are worth noting as they may account, at least partially, for the degree of gene expression regulation. The average running distance per week for chronic voluntary wheel exercised healthy animals was 6556 m/week, whereas, the average running distance per week for chronic voluntary wheel exercised diabetic animals was 5968 m/week. The diabetic runners' average weekly distance may be deceiving as the individual range was from 1785 m/week to 20,017 m/week, and group range was from 6000 m/week (week 1) to 2257 m/week (week 15) (ie. high variability). The range for the healthy voluntary wheel individual runners was from 4263 m/week to 9719 m/week, and the group range was from 4400 m/week (week 1) to 3508 m/week (week 16) (ie. relatively less variability). If we take away the two diabetic high distance runners from the average, the individual weekly running distance is now 4164 m/week (vs. 6556 m/week for the healthy runners). The total distance ran by the diabetic and healthy groups was 89,530m and 94,832m respectively (i.e. diabetic rats ran 5302m less in total as a group). The lesser distance of running by the diabetic animals may be due to various factors. However, one reason may be the rats' general ill health due to diabetes (i.e. general decline by the end of the 14th week of study, which most likely influenced running volume). Therefore, our results may have been different if this were not the case (i.e. with increased running volume we might expect even greater

differences in gene expression and behavioral changes compared to diabetic sedentary animals). Thirdly, differences in gene expression reflect thousands of neurons. Hence, an average response from a large number of neurons makes a higher p-value (i.e. trending or near statistical significance) actually very important (i.e. therefore genes that that did not differ statistically, may still be functionally relevant and worth exploring).

Recommendations for further study to continue these lines of research are numerous. Specific to study 2, an idea for future study would be to terminate STZ rats at 5 weeks and 9 weeks (ie. when behavioral testing occurred) to investigate gene expression at these time points, with and without exercise, so we can see what is happening with gene expression as diabetes and/or DPN progresses. Another similar study to study 2, with the addition of enhancement to general health (ie. low-dose insulin injection perhaps) post-diabetes induction, could result in higher volume of running and increase duration of study. This could potentially lead to greater differences and additions in gene expression changes, and would allow for the examination of DPN progression and determine whether or not exercise continues to prevent thermal hypoalgesia. This would also be more representative of the human condition as it is common for diabetics to have some sort of pharmaceutical treatment for diabetes management (especially type 1). In addition, one could examine the foot pads in these animals to see if there are changes to the number of epidermal nerve fibers. Further research is needed to determine if the prevention/reversal of thermal hypoalgesia is coherent with a prevention/reversal in cutaneous fiber loss in DPN. Also, to get a bigger picture of what is going on, it would help to perform muscle and nerve mRNA analysis for the genes that are altered in the DRG. Finally, establishing molecular mechanisms that may contribute to the beneficial effects experienced by diabetics who

exercise, could help with prescription for therapeutic and preventative benefits, while aiding in the knowledge of DPN pathogenesis.

Specific to study 1, it would be interesting to apply the results to a pain model to see the differences in gene expression (ie. we know exercise can help attenuate pain in certain pain conditions - do gene expression changes, in the 5HT1D receptor for example, accompany this behavioral change?). Clarification of functional properties of the associated differences in gene expression could possibly be provided with the addition of measurements taken during a movement/exercise as opposed to at rest after an exercise paradigm. Furthermore, studies addressing which receptors, metabotropic 5HT receptors in particular, are acting pre-synaptically, in spinal cord dorsal and ventral horns, to provide inhibitory or excitatory responses are needed. Since we excluded medium neurons in our studies, it would be valuable to isolate these cells and examine gene expression changes due to exercise. It would also be helpful to investigate small and large cell gene expression differences after 1 week of voluntary wheel exercise, as a comparison to the treadmill work, and to compare to whole DRG findings.

In conclusion, our results produce the first of its kind in terms of comparing large and small DRG cell gene expression after exercise. Small and large DRG neurons differ significantly in the expression of several genes investigated, as well as in their responsiveness to both acute and chronic exercise. Both amount and type of physical activity influence neuromuscular changes, and we did indeed show that voluntary wheel and treadmill trained animals differ in their expression of several genes in DRG neurons, as well as chronic exercise producing differences in gene expression compared to acute exercise. The DRG gene expression results, of exercise in healthy animals, provide a new potential mechanism of exercise analgesia, as well as proprioceptive functional changes such as improved motor coordination. The impact of exercise

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on gene expression, in the absence of some other intervention such as injury or disease, is a necessary baseline for future studies. Gene expression changes in healthy rats with voluntary wheel exercise do not seem to correspond to changes with exercise in diabetic rats. In voluntary wheel exercised diabetic rats, the differences in DRG gene expression provide a possible mechanistic insight into the prevention/reversal of thermal hypoalgesia. Further research is needed to determine if this change is long-term, and if it is consistent with a prevention in cutaneous fiber loss in DPN. The identification of affected and unaffected genes due to exercise and diabetes is useful to guide future studies.

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APPENDICES

Appendix 1: Solutions

STAINING CELLS FOR LCM

Cresyl violet method:

- 4% cresyl violet in 75% Ethanol = 500 mg /12.5 ml (75% Ethanol made with RNase free water)

- Let stir overnight with a sterile baked magnetic stir bar on a stir plate and then filter

- Keep in the fridge wrapped with foil as it is light sensitive

STZ PROTOCOL

The rats were fasted for 12-16 hours prior to the STZ injection. The morning of the STZ injections the rats were weighed, and these weights were used for the STZ injection calculations. A 1g bottle of STZ (Sigma: S0130-1G) was required for the number of rats in the study. The bottle was stored at -20°C. In the fume hood, we started by putting 10-15 ml of NaCl solution into the 15 ml tube, and put this on ice. Then we pre-filled 3 ml syringes with 0.9% NaCl (Baxter; JB1324) at room temperature. Next we took the STZ powder from the -20°C freezer and placed it on ice in the fume hood. We weighed out the STZ according to our calculations, and made up enough for 12 animals initially, and then did the other 12 later (since the STZ can deteriorate in as little as 30-45 minutes). We then dissolved the STZ in the 0.9% NaCl and used the vortex to mix for 1-2 minutes. Next, we inserted our needle directly into the rubber stopper of the vacutainer tube to draw out the STZ. Each animal was then given 90 mg/kg of dissolved STZ IP, followed immediately by 3 ml of 0.9% NaCl subcutaneously.

Appendix 2: qPCR protocols

RNA ISOLATION (based on 100ul of lysate solution)

*All solutions are proprietary and cannot be revealed.

- Warm wash solution 2/3 to room temperature.

- Pre-wet micro filter using 30ul lysis solution for 5 minutes – centrifuge 30 seconds at 13000rpm

- Add 3ul LCM additive to lysate – mix by vortexing and centrifuge.

- For RNA > 75 bases: add 0.5 volume = 52ul 100% ethanol and pipette up and down to mix.

- Load entire lysate/ethanol unto prepared micro filter – centrifuge 1minute at 10300rpm.

- Wash with 180ul solution 1 – centrifuge 1 minute at 10300rpm.

- Add 180ul solution 2/3 (RT) – centrifuge 30seconds at max speed

- Repeat the above step

- Pour out flow thru.

- Replace micro filter in same collection tube and centrifuge 30seconds at maximum speed to remove residual.

- Transfer filter to micro elution tube.

- Apply 10ul elution solution, preheated to 95°C, to center of filter. Close cap and store 5 minutes at RT.

- Centrifuge 1 minute at 10000rpm to elute RNA.

- Add another 8ul of elution solution and repeat and repeat above step (ie. to give higher concentration – only need 14ul to make cDNA).

- Put flow thru in small tube.

- Add 1/10th volume DNASE Buffer (2ul in 20ul RNA eulted) + 1ul DNase (mix all gently – do not vortex).

- Incubate 20mins at 37°C.

- Thaw DNAse inactivation reagent and vortex. Use 2ul or 1/10th (highest of 2 values) volume DNAse inactivation reagent.

- Let sit 2 minutes – hand vortex.

- Centrifuge 1.5 minutes at 13000rpm.

- Transfer to RNAse free tube and store at -80°C

AGILENT BIOANALYZER (Agilent RNA 6000 Pico Kit)

- We prepared the assay by:
  - 1) Prepare the gel: Allow all reagents to equilibrate to room temperature for 30 minutes before use. Place 550 μl of RNA 6000 Pico gel matrix into the top receptacle of a spin filter. Place the spin filter in a microcentrifuge and spin for 10 minutes at 1500 g ± 20 %. Aliquot 65 μl filtered gel into 0.5 ml RNase-free microcentrifuge tubes that are included in the kit. Store the aliquots at 4 °C and use them within one month of preparation.
  - 2) Prepare the Gel-Dye mix: Allow all reagents to equilibrate to room temperature for 30 minutes before use. Protect the dye concentrate from light while bringing it to room temperature. Vortex RNA 6000 Pico dye concentrate for 10 seconds and spin down. Add 1 μl of RNA 6000 Pico dye concentrate to a 65 μl aliquot of filtered gel. Cap the tube, vortex thoroughly and visually inspect proper mixing of

gel and dye. Store the dye concentrate at 4 °C in the dark again. Spin tube for 10 minutes at room temperature at 13000 g. Use prepared gel-dye mix within one day.

- o 3) Load the gel-dye mix.
- o 4) Load the RNA 6000 Pico conditioning solution and marker.
- o 5) Load the diluted ladder and samples

- Insert chip into Biolanalyzer and start chip run. The analysis run time was 30 minutes with 11 samples per chip.

cDNA SYNTHESIS

- Combine the following components in a tube on ice: 4ul of 5x VILO Reaction Mix; 2ul of 10x SuperScript Enzyme Mix; Xul of RNA (up to 2.5 ug); to 20ul of DEPC-treated water

- Gently mix tube contents and incubate at 25°C for 10 minutes.

- Incubate the tube at 42°C in a thermal cycler for 60 minutes, then 85°C for 5 minutes before terminating the reaction.

- Store cDNA at -20°C until use, or proceed to pre-amplify.

Pre-amplification Procedure:

- The pre-amplification reactions were mixed as follows: 25ul of TaqMan PreAmp Master Mix (2X), 12.5ul of Pooled assay Mix (0.2X, each assay) and 12.5ul of 1-250 ng cDNA sample plus nuclease free water.

- Once mixed, the reaction proceeded as follows:

	HOLD	14 CYCLES	
Temperature	95 °C	95 °C	60 °C
Time	10 minutes	15 seconds	4 minutes

- Once the reaction was complete, we immediately removed the tube from the thermal cycler (Eppendorf Mastercycler Model 5331 H48014) and placed it on ice.

- Lastly, we diluted (1:20 with 1XTE Buffer) the pre-amplification product and stored it at -20 °C.

RT-qPCR PLATE PREPARATION

The PCR plates were filled as follows: 20ul of GEA Master Mix per well (ie. we used triplicate format, hence, 24 wells per each GEA). The Master Mix was composed of 1.25ul of GEA, 6.25ul of water and 12.5ul of master assay mix. Then we added 5ul of cDNA per well. Once the plate was complete, we centrifuged it at 2500 RPM for 2 minutes.

Appendix 3: Apparatus Specifications

Lafayette Voluntary Wheel specifications:

- Tub Dimensions: 16" x 20" x 8.25"H (40.64 x 50.80 x 20.96 cm)

- Overall Dimensions: 16" x 20" x 20.5"H& (40.64 x 50.80 x 52.07 cm)

- Weight: 15 lbs (6.8 kg)

- Wheel Diameter: 18" (45.7 cm) I.D.

- Wheel Width: 5.25" (13.3 cm) internal

- Run Distance: ~1.44 meter per revolution

Columbus treadmill:

- Overall dimensions: 33" x 20" x 20" (33 x 50.8 x 50.8cm)

- Each exercise lane dimensions:

    o Rats: 17.25"L x 4.75"W x 5"H (43.8 x 12 x 12.7 cm)

- Stimulus area (shocking grid area):

    o Rats: 4.5" x 4.75" (11.4 x 12cm)

- Stimulus current: 0 to 1.5mA (adjustable)

- Stimulus voltage: 163V

- Tread inclination: 0 to 25° in 5° steps

Appendix 4: Surgical Procedures

SPINAL CORD IN SUCROSE (identifying rat DRGs and dorsal roots):
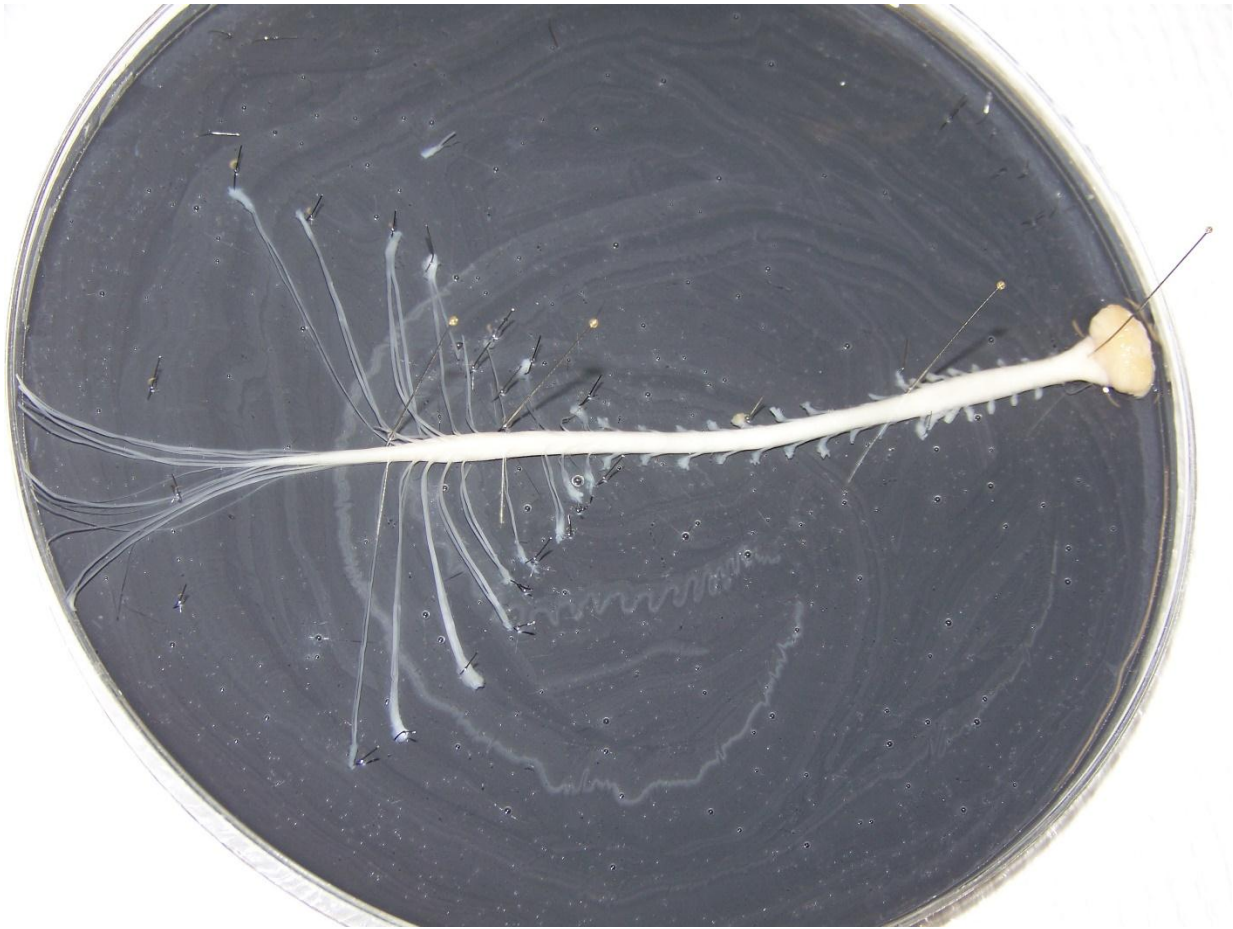
DRG REMOVAL METHOD:

1. Remove the spinal column from the neck to the base of the tail

2. Starting at the proximal end of the spinal column, remove a section in the shape of a V, from the dorsal portion of the vertebrae

3. Cut the spinal column in half longitudinally through the ventral vertebrae; Using Dumont #5 forceps, remove the DRGs from each half of the spinal column

Appendix 5: Exercise Data

Healthy rat voluntary wheel running weekly data (average)

WEEK	Rat ID									Avg distance (m)
	1	2	3	4	5	6	7	8		
1	5342	6679	3630	4212	5233	2185	6971	950		4400
2	10448	12695	9594	7116	13040	8185	13315	2768		9645
3	9630	15473	8844	6592	14380	5019	5129	6444		8939
4	8640	12506	12083	6325	15084	11137	13328	10909		11252
6	6488	11536	6678	5018	11573	7738	8397	10873		8538
7	6133	8178	6339	4353	12507	6633	8567	9473		7773
8	4481	8995	1187	3959	11663	5030	6032	8184		6191
9	4052	10068	8134	3151	9457	5597	4920	7311		6586
10	4054	9960	8336	3410	11368	4967	4949	8035		6885
11	4034	9187	8012	3561	9780	3382	5315	8321		6449
12	3505	6982	6287	4839	8170	2268	4412	6519		5373
13	3333	6774	4887	3876	7651	2808	4669	6358		5044
14	3896	5507	3623	2831	5710	1875	4299	6721		4308
15	3105	4395	3473	2403	5464	1652	2550	4549		3449
16	3233	4857	3869	2299	4710	1718	2859	4521		3508
Avg distance (m)	5358	8920	6332	4263	9719	4680	6381	6796	6556	6556
Sum distance (m)	80375	133793	94976	63946	145789	70195	95711	101935	98340	98340

Diabetic rat voluntary wheel running weekly data (average)

WEEK	STZ RAT ID													Avg distance (m)
	1	2	3	4	5	6	7	8	9	10	11	12		
1	4076	4458	3950	9913	2903	13138	10256	5146	3697	4706	2762	6993		6000
2	4215	5602	3387	12368	1553	19402	12868	4894	2645	2286	2010	9288		6710
3	3350	11358	1940	31323	2357	38093	23779	10199	5293	3700	3252	8974		11968
4	3681	11935	2903	30987	1834	37446	19630	10782	4231	2536	3105	7304		11364
5	2982	7221	2993	34489	2283	8089	16825	11605	5649	2018	4566	5801		8710
6	1446	3081	1284	15798	1742	4237	4929	7098	2845	720	2415	2206		3983
7	2264	3518	1762	20626	1643	5160	3383	10438	4080	1995	3854	4092		5235
8	2376	3308	2409	25972	2120	7683	4049	13198	5330	1873	3900	6106		6527
9	2990	2427	2230	24325	1748	3146	4174	8388	3006	1108	3586	5476		5217
10	2063	2337	2757	18110	1425	5560	3810	10457	2695	1153	2056	4974		4783
11	1508	1813	2636	19204	1465	4693	2085	8830	3137	842	1187	4017		4285
12	1927	1239	2828	25136	1383	2584	5181	8864	3633	1158	1995	3961		4991
13	1815	1878	2170	12551	1397	2924	5280	6602	5582	517	1936	7435		4174
14	1001	2224	1832	13431	1273	5195	2092	5905	4120	379	313	2154		3326
15	sac	sac	745	6012	sac	sac	1310	sac	sac	sac	sac	962		2257
Avg distance (m)	2550	4457	2389	20016	1795	11239	7977	8743	3996	1785	2638	5316	6075	5969
Sum distance (m)	35693	62397	35828	300247	25126	157349	119648	122404	55942	24990	36936	79741	88025	89530

Appendix 6: Average RQ values

	SMALL		LARGE	
Gene	Sedentary (n=5)	Treadmill (n=6)	Sedentary (n=5)	Treadmill (n=5)
ION CHANNELS				
$Na_v1.9$	6086.17 ± 1055.35	6041.96 ± 1513.07	713.44 ± 16.84	736.72 ± 158.15
$K_{Ca}2.3$	3.66 ± 0.42	3.73 ± 0.85	2.63 ± 0.66	2.53 ± 0.53
$K_v1.2$	1.03 ± 0.16	1.13 ± 0.18	2.03 ± 0.31	1.89 ± 0.15
$K_v1.1$	0.70 ± 0.12	0.62 ± 0.13	1.58 ± 0.57	1.58 ± 0.59
$K_{Ca}2.2$	0.70 ± 0.16	0.75 ± 0.17	2.00 ± 0.77	1.82 ± 0.57
$Na_v1.6$	0.68 ± 0.13	0.65 ± 0.24	2.27 ± 0.39	2.14 ± 0.16
$Na_v1.2$	0.12 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.08 ± 0.01
GABA & ADRENERGIC RECEPTORS				
$GABA_{A\gamma2}$	5.33 ± 1.15	5.66 ± 1.30	4.85 ± 1.17	6.04 ± 1.23
$GABA_{B2}$	3.77 ± 0.96	3.93 ± 0.99	2.83 ± 0.70	2.85 ± 0.23
$GABA_{A\alpha2}$	2.11 ± 0.52	1.92 ± 0.61	2.51 ± 0.44	2.60 ± 0.20
$GABA_{B1}$	1.36 ± 0.22	1.39 ± 0.32	1.22 ± 0.27	1.10 ± 0.14
$GABA_{A\beta3}$	1.33 ± 0.25	1.34 ± 0.16	1.95 ± 0.37	1.95 ± 0.16
$ADRA_{A1\alpha}$	0.04 ± 0.02	0.04 ± 0.01	0.30 ± 0.07	0.25 ± 0.09
$ADRA_{A1\delta}$	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
GLUTAMATERGIC RECEPTORS				
$NMDAR_1$	0.72 ± 0.24	0.68 ± 0.18	0.40 ± 0.06	0.37 ± 0.05
Glur2	0.40 ± 0.06	0.37 ± 0.07	0.57 ± 0.09	0.55 ± 0.06
Glur3	0.09 ± 0.01	0.08 ± 0.01	0.20 ± 0.04	0.19 ± 0.01
5HT RECEPTORS				
$5HT_{1A}$	0.68 ± 0.22	1.06 ± 0.37	0.29 ± 0.13	0.41 ± 0.08
$5HT_7$	0.60 ± 0.06	0.63 ± 0.08	1.20 ± 0.26	1.10 ± 0.22
$5HT_{2A}$	0.55 ± 0.08	0.54 ± 0.06	0.41 ± 0.04	0.38 ± 0.04
$5HT_{2C}$	0.01 ± 0.00	0.01 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
TYROSINE KINASE RECEPTORS				
TrkA	189.71 ± 52.10	194.77 ± 25.98	107.13 ± 20.02	104.16 ± 17.93
TrkC	1.27 ± 0.34	1.20 ± 0.29	5.16 ± 1.00	5.16 ± 0.60
TrkB	0.62 ± 0.39	0.51 ± 0.27	0.72 ± 0.12	0.84 ± 0.18
GROWTH RELATED				
BDNF	10.72 ± 2.83	11.54 ± 4.24	7.57 ± 1.69	8.48 ± 1.49
GAP43	4.14 ± 0.85	3.93 ± 0.84	1.85 ± 0.38	1.99 ± 0.34

NT3	1.14 ± 1.39	1.67 ± 1.74	1.22 ± 1.67	0.86 ± 0.68
NGF	0.48 ± 0.07	0.46 ± 0.15	0.44 ± 0.18	0.36 ± 0.13
SYNAPTIC VESICLE RELATED				
STX1A	6.86 ± 1.09	5.64 ± 1.58	1.04 ± 0.13	1.01 ± 0.28
VGlut2	2.09 ± 0.34	2.01 ± 0.55	0.90 ± 0.09	1.04 ± 0.19
SYN1	1.76 ± 0.26	2.02 ± 0.30	1.35 ± 0.19	1.30 ± 0.14
STX1B	0.39 ± 0.07	0.37 ± 0.07	0.53 ± 0.35	0.49 ± 0.32

Key:
Average RQ value ± standard deviation
Average RQ values reflect mRNA abundance

Study 1 – Chronic treadmill and voluntary wheel exercise

	SMALL			*LARGE*		
Genes	Sedentary (n=4)	Treadmill (n=5)	Voluntary Wheel (n=7)	Sedentary (n=5)	Treadmill (n=5)	Voluntary Wheel (n=7)
ION CHANNELS						
$Na_v1.9$	11840.22 ± 2713.85	10873.46 ± 1535.26	11086.92 ± 2814.00	1712.04 ± 845.86	1664.44 ± 681.76	2002.40 ± 1199.42
$Na_v1.8$	6034.86 ± 799.00	6195.15 ± 2166.94	5986.73 ± 1497.83	1574.41 ± 588.71	1465.97 ± 184.77	1648.74 ± 499.78
TRPV1	238.82 ± 41.53	258.90 ± 15.84	256.80 ± 38.93	38.15 ± 18.35	35.84 ± 15.74	53.34 ± 24.71
$Na_v1.7$	74.72 ± 9.42	86.49 ± 12.41	63.98 ± 14.68	41.31 ± 7.73	48.75 ± 13.69	41.39 ± 8.55
$Na_v1.6$	0.84 ± 0.17	0.79 ± 0.15	0.74 ± 0.14	2.72 ± 0.27	2.35 ± 0.36	2.24 ± 0.28
$Na_v1.3$	0.43 ± 0.09	0.38 ± 0.02	0.50 ± 0.08	0.13 ± 0.04	0.12 ± 0.02	0.17 ± 0.06
$Na_v1.2$	0.13 ± 0.02	0.11 ± 0.02	0.12 ± 0.03	0.10 ± 0.02	0.09 ± 0.02	0.09 ± 0.02
5HT RECEPTORS						
$5HT_{3A}$	49.32 ± 6.78	52.40 ± 7.76	54.35 ± 7.65	30.19 ± 5.38	29.73 ± 3.97	40.50 ± 8.40
$5HT_{1D}$	22.10 ± 2.82	72.68 ± 7.94	12.10 ± 2.55	71.38 ± 9.88	65.97 ± 4.30	41.98 ± 3.48
$5HT_{1B}$	1.61 ± 0.46	1.57 ± 0.29	1.34 ± 0.34	3.36 ± 0.68	3.42 ± 0.71	2.87 ± 1.25
$5HT_{1A}$	1.11 ± 0.35	0.65 ± 0.17	0.95 ± 0.29	0.68 ± 0.33	0.20 ± 0.08	0.40 ± 0.18
$5HT_{1F}$	0.62 ± 0.12	0.21 ± 0.04	0.90 ± 0.27	0.37 ± 0.13	0.29 ± 0.06	0.47 ± 0.17
TYROSINE KINASE RECEPTORS						
TrkA	147.42 ± 20.30	149.05 ± 31.50	89.09 ± 28.58	105.38 ± 13.93	90.80 ± 12.61	67.42 ± 15.26
TrkC	1.28 ± 0.28	1.31 ± 0.25	1.17 ± 0.29	6.06 ± 0.92	4.54 ± 0.62	4.49 ± 0.48
TrkB	0.85 ± 0.08	0.75 ± 0.05	0.91 ± 0.08	0.80 ± 0.09	0.65 ± 0.09	0.76 ± 0.11
GROWTH RELATED GENES						

Gene						
BDNF	7.06 ± 0.83	8.56 ± 2.49	9.06 ± 3.39	4.26 ± 2.05	7.04 ± 1.39	7.62 ± 3.76
GAP43	4.13 ± 0.46	3.58 ± 0.49	4.99 ± 0.20	1.76 ± 0.58	1.92 ± 0.24	2.62 ± 0.37
IGF1	1.38 ± 0.54	1.29 ± 0.52	1.53 ± 0.42	1.33 ± 0.63	1.07 ± 0.31	1.55 ± 0.44
NT3	0.94 ± 0.58	0.78 ± 0.28	0.83 ± 0.46	0.68 ± 0.42	0.46 ± 0.12	0.93 ± 0.56
NGF	0.38 ± 0.10	0.46 ± 0.16	0.59 ± 0.14	0.41 ± 0.21	0.24 ± 0.15	0.57 ± 0.18
SYNAPTIC VESICLE RELATED GENES						
STX1A	7.16 ± 1.34	6.64 ± 0.51	7.07 ± 0.60	1.39 ± 0.70	1.46 ± 0.32	1.77 ± 0.76
SYN1	2.09 ± 0.29	2.10 ± 0.53	1.99 ± 0.25	2.04 ± 0.37	1.58 ± 0.22	1.47 ± 0.13
PAIN RELATED GENES SubP	24.14 ± 1.87	28.71 ± 5.93	25.14 ± 10.63	3.45 ± 1.65	3.83 ± 1.54	4.65 ± 2.06
CGRP	21.55 ± 5.34	19.88 ± 3.45	18.84 ± 4.91	5.90 ± 2.36	6.66 ± 1.22	8.10 ± 2.56
OPRM1	5.64 ± 0.82	6.39 ± 0.94	7.28 ± 1.47	1.86 ± 0.76	1.45 ± 0.23	2.63 ± 0.62
OPRD1	0.71 ± 0.09	0.56 ± 0.10	0.41 ± 0.19	3.05 ± 0.87	2.24 ± 0.43	2.06 ± 0.46
CNR1	0.10 ± 0.02	0.10 ± 0.04	0.10 ± 0.02	0.30 ± 0.06	0.22 ± 0.04	0.27 ± 0.07

Key:
Average RQ value ± standard deviation
Average RQ values reflect mRNA abundance

Study 2 – Diabetes and voluntary wheel exercise

	SMALL			*LARGE*		
Gene	Healthy Sedentary (n=6)	Diabetic Sedentary (n=10)	Diabetic Voluntary Wheel (n=12)	Healthy Sedentary (n=6)	Diabetic Sedentary (n=10)	Diabetic Voluntary Wheel (n=12)
ION CHANNELS						
TRPV1	2.32 ± 0.38	2.20 ± 0.26	2.30 ± 0.32	0.22 ± 0.10	0.25 ± 0.14	0.22 ± 0.07
$Na_v1.8$	1.41 ± 0.20	1.33 ± 0.16	1.26 ± 0.19	0.40 ± 0.12	0.36 ± 0.10	0.31 ± 0.06
5HT RECEPTORS						
$5HT_{1A}$	2.45 ± 0.81	2.79 ± 0.67	1.95 ± 0.64	0.94 ± 0.51	1.16 ± 0.56	0.85 ± 0.61
$5HT_{1F}$	1.12 ± 0.23	1.46 ± 0.10	1.43 ± 0.34	0.78 ± 0.33	0.95 ± 0.22	0.92 ± 0.33
TYROSINE KINASE RECEPTORS						
TrkA	1.56 ± 0.37	1.43 ± 0.16	1.39 ± 0.36	0.97 ± 0.13	0.91 ± 0.20	0.82 ± 0.11
TrkB	0.83 ± 0.08	0.73 ± 0.05	0.79 ± 0.07	0.68 ± 0.06	0.73 ± 0.20	0.67 ± 0.05
TrkC	0.29 ± 0.10	0.34 ± 0.08	0.35 ± 0.10	1.56 ± 0.25	1.61 ± 0.21	1.67 ± 0.21

GROWTH RELATED GENES						
BDNF	2.01 ± 0.68	1.44 ± 0.36	1.75 ± 0.53	1.61 ± 0.57	1.83 ± 0.50	2.52 ± 1.14
GAP43	1.63 ± 0.15	1.70 ± 0.20	1.62 ± 0.18	0.86 ± 0.17	0.77 ± 0.12	0.79 ± 0.12
IGF1	0.85 ± 0.25	0.98 ± 0.31	0.73 ± 0.22	0.85 ± 0.21	0.75 ± 0.15	0.79 ± 0.28
GDNF	0.78 ± 0.12	0.84 ± 0.28	0.84 ± 0.24	0.29 ± 0.20	0.34 ± 0.17	0.46 ± 0.26
NT3	0.55 ± 0.24	1.96 ± 1.40	1.47 ± 1.47	0.65 ± 0.57	0.82 ± 0.36	1.01 ± 0.66
NGF	0.30 ± 0.20	0.32 ± 0.07	0.33 ± 0.08	0.16 ± 0.06	0.21 ± 0.06	0.22 ± 0.08
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SubP	3.74 ± 1.29	3.23 ± 0.67	3.28 ± 1.02	0.40 ± 0.33	0.28 ± 0.16	0.28 ± 0.07
CGRP	2.95 ± 0.95	2.21 ± 0.24	2.08 ± 0.61	0.68 ± 0.60	0.44 ± 0.11	0.50 ± 0.14

Key:
Average RQ value ± standard deviation
Average RQ values reflect mRNA abundance

Appendix 7: Power Analysis

Power analysis was conducted to determine sample sizes for each experiment. The

following equation was used (Hassard, T., 1991): $n = (PI \times \sigma/\mu_d)^2$

$n = (3.24 \times 22.3/62.5)^2$
$n = 1.35$

Where:

n = required number of subjects

PI = power index, calculated from the desired power of the study (3.24; based on alpha and beta error assumptions of 0.05 (5% chance of committing a Type I error e.g. failure to detect a real difference) and 0.10 (10% chance of committing a Type II error e.g. detecting a difference when none exists) respectively)

μ_d = true mean difference between two different treatment methods

σ = true standard deviation of the difference between two different treatment method

Generally, SD is approximately on average 22.3% of the average mean difference (62.5% change) in this type of experiment (Molteni et al, 2004; Fig. 4). When using these values, based on the equation above, we obtain an n-value of 1.35. Thus, our study required a sample size of at least 2 animals, but based on the novelty of our particular experiments, we used a minimum n-value of 5 animals per study.